CELL DEFORMABILITY AND THE SEQUESTRATION OF NEUTROPHILS IN THE LUNGS DURING CIGARETTE SMOKING

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I declare that this thesis was written by me
and that the work contained herein is my own except where otherwise
indicated

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ABSTRACT

Cigarette smoking is a major cause of the chronic lung disorder, emphysema. The pathogenesis of emphysema is hypothesised to result from a proteases/antiproteases imbalance in peripheral lung areas allowing proteolytic destruction of the connective tissue matrix of the alveolar walls. As chronic cigarette smoking is associated with an increased number of blood and alveolar leucocytes, these cells have been implicated as important sources of such proteolytic enzymes. Neutrophil transit through the pulmonary circulation is delayed in comparison to the transit of erythrocytes. During inhalation of cigarette smoke, this delay or sequestration of neutrophils, which occurs in normal lungs, was found to be enhanced. This could facilitate neutrophil emigration into the airspace or activation of sequestered neutrophils could result in an elastase burden in the pulmonary vasculature. Hence, studies of the mechanism of the enhanced neutrophil sequestration during smoking would improve our knowledge and may lead to therapeutic interventions for smoke-induced lung diseases.

The intravascular pulmonary sequestration of neutrophils is influenced by haemodynamic forces, the cell's ability to deform, and by an increase in adhesion between neutrophils and endothelial cells. This thesis has concentrated on the influence of cell deformability on neutrophil sequestration in the lungs and the effects of cigarette smoking.

An *in vitro* measurement of cell deformability was compared to the *in vivo* kinetics of neutrophils in the lungs of man, to reveal a strong correlation between the first pass sequestration *in vivo* and *in vitro* cell deformability. Exposure of isolated neutrophils to the vapour phase of cigarette smoke *in vitro* caused a reduction in cell deformability. However, recovery of cell deformability was observed with time. The functional behaviour of smoke exposed neutrophils was also adversely affected. The mechanism for the reduction in cell deformability on exposure to cigarette smoke appeared to be due to the oxidant effect of smoke on the cell cytoskeleton.
Moreover, in confirmation, a reduction in leucocyte deformability was observed in arterial but not venous blood, by measuring the filterability of diluted whole blood, following *in vivo* cigarette smoking .

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Selby C, Drost E, Wraith PK and MacNee W. *In vivo* neutrophil sequestration within lungs of humans is determined by *in vitro* "filterability". J Appl Physiol 1991; 71: 1996-2003.

Brown GM, Drost E, Donaldson K, McGregor I and MacNee W. Reduction of the proteolytic activity of neutrophils by exposure to cigarette smoke *in vitro*. Exp Lung Res. 1991; 17: 923-937.

Lannan S, McLean A, Drost E, Gillooly M, Donaldson K, Lamb D and MacNee W. Changes in neutrophil morphology and morphometry following exposure to cigarette smoke. Intern J Pathol 1992; 73: 183-191.

ABBREVIATIONS

CELL HARVESTING

ACD	Acid citrate dextrose anticoagulant
BSA	Bovine serum albumin
EDTA	Ethylene diamine tetra-acetic acid
LPS	Lipopolysaccharide, endotoxin
MNL	Mononuclear leucocyte
PBS	Phosphate buffered saline
PMN	Polymorphonuclear leucocyte, neutrophil
PPP	Platelet poor plasma
RBC	Red blood cell, erythrocyte

CELL FUNCTION

CD	Cluster of differentiation number (of leucocyte antigens)
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
H_2O_2	Hydrogen peroxide
ICAM	Intercellular adhesion molecule
NBT	Nitro Blue tetrazolium
O_2^-	Superoxide anion
PMA	Phorbol Myristate acetate
SOD	Superoxide dismutase

CELL STRUCTURE

ADP	Adenosine diphosphate
AFC18	5-N-(octadecanoyl) aminofluorescein
ATP	Adenosine 5'-triphosphate
CB	Cytochalasin
CSC	Cigarette smoke condensate
DMSO	Dimethyl sulfoxide
F-actin	Filamentous actin
FRAP	Fluorescence recovery after photobleaching
G-actin	Globular actin
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HOCl	Hypochlorous acid
MDA	Malondialdehyde
NBDPC	1-hexadecanoyl-2-[N-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl]-sn-glycero-3-phosphocholine
NBD phallacidin	N-7-nitrobenz-2-oxa-1,3-diazol-4-yl- phallacidin
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

CELL DEFORMABILITY

From the viscoelastic solid model of neutrophil deformation:

k_1	Elastic spring ($dyn.cm^{-2}$)
k_2	Elastic element ($dyn.cm^{-2}$)
μ	Viscous element ($dyn.s.cm^{-2}$)
P_6	Pressure developed following 6 minutes filtration (cm H_2O)
P_{grad}	Initial pressure gradient upon filtration ($cmH_2O.min^{-1}$)

CLINICAL

α_1Pi	Alpha$_1$-proteinase inhibitor
COHb	Carboxyhaemoglobin as percent of total
COPD	Chronic obstructive pulmonary disease
FEV_1	Forced expiratory volume in 1 second (Litre)
FVC	Forced vital capacity (Litre)
H^+	Arterial blood hydrogen ion concentration (nM)
$PaCO_2$	Arterial blood carbon dioxide tension (kPa)
PaO_2	Arterial blood oxygen tension (kPa)
%Pred	Percentage of that predicted
WBC	White blood count ($x10^9.L^{-1}$)

***IN VIVO* KINETIC STUDIES**

^{111}In	Indium-111
^{99m}Tc	Technicium-99m
10's	Neutrophil lung sequestration at 10 minutes after reinjection as a percentage of the first minute
C	Rate constant for ^{111}Indium time/ activity monoexponential curve ($x10^{-3}.s^{-1}$)
INS	Normalised initial sequestration of neutrophils on their first lung passage relative to that of erythrocytes (ratio)
KeV	Kiloelectron-volt, where 1 KeV is the energy acquired by the electron when the potential difference through which the electron accelerates is 1 volt
MBq	Megabecquerel, the amount of radioactive material in which 1 atom disintegrates per second
ROI	Region of interest from which time/ activity curves were obtained

CHAPTER 1
INTRODUCTION

1 INTRODUCTION

A study performed by MacNee and colleagues in 1989 (1989d) demonstrated an enhanced delay in the transit of radiolabelled neutrophils in the pulmonary circulation during acute smoking. By comparison, smokers who did not actively smoke showed only the normal delay in neutrophil transit relative to the passage of erythrocytes in the pulmonary circulation (MacNee, 1989d). Activation of these neutrophils sequestered in the pulmonary microvasculature of smokers, inducing the release of toxic radicals and proteases, could result in lung damage. A chronic cigarette smoking-related lung disease, pulmonary emphysema, is proposed to develop as a result of a protease/antiprotease and/or an oxidant/antioxidant imbalance (Janoff, 1983a). Hence, knowledge of the mechanism of neutrophil sequestration in the lungs during the act of smoking could be beneficial in understanding the pathogenesis of emphysema and also other smoke related diseases, and in developing therapies for these conditions. In this thesis the hypothesis tested was that smoke-induced neutrophil sequestration was due to an effect on neutrophil rheology. Hence, the effect of *in vitro* and *in vivo* cigarette smoke exposure on neutrophil deformability was investigated.

The following introduction reviews the neutrophil and its association with lung injury in general, and the evidence that cigarette smoking can be detrimental to the lung. Also the major factors which influence neutrophil transit in the lungs are compared to examine the likelihood of a change in cell deformability causing leucocyte lung sequestration during smoking.

1.1 THE STRUCTURE AND FUNCTION OF THE LUNGS

1.1.1 THE RESPIRATORY SYSTEM

The mammalian respiratory system comprises a system of airway tubes, peripheral airspaces and a system of blood vessels which converge in the lungs to allow gas exchange. The airways from the proximal to the distal lung consist of the trachea which branches like a tree into bronchi, bronchioles, and terminal bronchi (the conducting airways) which branch further into the respiratory bronchioles, alveolar ducts and alveoli (the respiratory airways). The lung unit distal to the terminal bronchi, termed the acinus, is the site of gas exchange (Weibel, 1984).

1.1.2 THE PULMONARY VASCULATURE

The vasculature of the lungs has two distinct systems. A high pressure systemic system, supplied from the aorta and the bronchial circulation which nourishes the lung tissues, and the lower pressure pulmonary circulation which perfuses the alveolar capillaries with blood and takes part in gas exchange. On the venous side, the capillaries of the bronchial circulation merge into the post capillaries which join to form venules that drain the blood into veins. The aorta, bronchial arteries, and arterioles are surrounded by a sleeve of smooth muscle which can contract to regulate blood flow. The aorta and arteries are also particularly elastic to allow absorption of pulses of blood pumped from the ventricle. The branching nature of the vasculature also ensures that the haemodynamic pressure is reduced enough to avoid injury to the delicate capillaries which consist of only a thin continuous endothelial cell layer and a basement membrane, and are separated from the alveolar space by a thin and continuous epithelial cell layer. However, pressure must be high enough for blood to reach the capillaries. This can be achieved by keeping the vessel diameter large. However, too large a diameter would require more energy, placing excessive stress on the heart. Hence, in this well designed system, the largest pressure drop occurs in the arterioles, the last segment prior to the capillary bed. The capillary wall structure is minimal in order to allow peripheral gas exchange in the tissues, but continuous to confine the blood to the vascular compartment and yet able to withstand the small positive pressure of blood which flows through it (Weibel, 1984).

The pulmonary vascular network of the lung is very different from that of the systemic bronchial circulation. Firstly, the pressures in the pulmonary circulation differ from that of the systemic circulation. Pressures in the pulmonary microcirculation are an order of magnitude lower than those in the systemic circulation (Nagasaka, 1984; von Euler, 1947; Weibel, 1984). Moreover, blood flow is pulsatile (de Lee, 1955; Weibel, 1984), which may contribute to a "stop-go" transit of leucocytes (Lien, 1990). Thus, as blood flow is the only pressure available to drive blood cells through capillary segments, their transit through the pulmonary circulation may be very different than transit through systemic microvasculature.
Secondly, the mean pulmonary capillary diameter is 5 μm (range 1-16 μm)(Guntheroth, 1982; Weibel, 1963), which is less than the mean systemic capillary diameter which is reported to be 6 μm (Folkow, 1971). Moreover, the capillary diameter in the lungs can be further reduced by a combination of a high alveolar pressure and low vascular pressure (Glazier, 1969). West (1964) described 3 zones

in the lungs to account for the distribution of blood flow in the lungs. Zone I, at the apex of the lungs, has a hydrostatic pressure (alveolar pressure and the effect of gravity on blood) which exceeds the inflow pulmonary arterial pressure which is low as the blood must be pumped against a pressure head from the heart (located mid-height of the lungs) to the apex of the lungs (Weibel, 1984). Therefore, for zone I conditions the pulmonary capillaries are narrowed or closed due to an excess hydrostatic pressure (Perlo, 1975; West, 1964). In zone II the haemodynamic pressure is greater than hydrostatic pressure allowing blood flow. However, as the outflow pressure in the veins is below atmospheric pressure, the capillaries can collapse. An increase in haemodynamic pressure and hence blood flow in this zone is reported to occur by recruitment of the closed vessels (Perlo, 1975; West, Dollery, 1964). In zone III conditions, at the base of the lungs, venous blood pressures are higher than alveolar pressures and flow is determined by the arterio-venous pressure difference. With an increase in pressure, the capillaries in zone III are further distended (Perlo, 1975; West, 1964).

Thirdly, the pulmonary capillaries consist of very short interposing tubular segments which contrast with the longer parallel interconnections of the systemic capillaries. Weibel (1963) estimated there to be approximately 277×10^9 capillary segments in the human lung with a mean length of 8 μm (range 1-30 μm). Weibel also determined the presence of around 296×10^6 alveoli, producing of the order of 1000 capillary segments per alveolus. Taking the distance from the arterial to the venous end of the capillary bed to be approximately 800 μm, as reported in the rabbbit, dog and cat (Staub, 1968), Hogg (1987) calculated blood cells must pass on average through approximately 100 capillary segments from arteriole to venule.

1.2 BLOOD CELLS

Blood is a suspension of red blood cells (erythrocytes), white blood cells (polymorphonuclear and mononuclear leucocytes), and platelets in plasma. All blood cells are derived from a common haematopoeitic pluripotent stem cell, generated chiefly in the myeloid and lymphatic tissues in the adult. The stem cells are stimulated to proliferate and differentiate, by the influence of colony stimulating factors, to progenitors of erythrocytes, granulocytes, macrophages, and lymphocytes. Moreover, changes in the levels of these specific glycoproteins in the enviroment of mature cells, such as may occur in response to inflammation or the stress caused by major surgery and trauma, can cause functional activation of the leucocytes (Lopez, 1986).

The white cell series is comprised of five different cell types, which can be classified according to their light microscopic appearance into two main classes: polymorphonuclear and mononuclear leucocytes. The polymorphonuclear leucocytes or granulocytes are characterised by a single, but multi-lobed nucleus and prominent cytoplasmic granules of which there are two types: the azurophilic or primary granules which contain lysozymes and are present in all granulocytes, and specific or secondary granules which are specific to each cell type. The staining characteristics of these specific granules allow the further classification of the different granulocytes into: eosinophils, which are stained by strong acidic dyes such as eosin; basophils which are stained with basic dyes such as methylene blue; and neutrophils which have little affinity for either. The mononuclear leucocytes contain non-segmented nuclei and no cytoplasmic granules. They compromise the monocytes, which migrate out of the blood stream into the tissues where they mature into macrophages, and the lymphocytes. The granulocytes and monocytes are active phagocytes, engulfing and digesting micro-organisms and debris in the blood and tissues. The lymphocytes play a key role in immune responses, directed specifically against foreign agents.

1.2.1 NEUTROPHIL STRUCTURE AND FUNCTION.

Neutrophils, monocytes and macrophages, with an arsenal of anti-microbial substances, are highly developed to destroy foreign material. The neutrophil's armamentarium is generated from two locations within the cell. The membrane associated systems generate reactive oxygen metabolites, and the cytosolic system of intracellular granules, termed lysosomes, contain potent proteinases and other enzymes. In the process of phagocytosis neutrophil pseudopods adhere and enclose the foreign object into a vacuole, called a phagosome. This phagosome then fuses with a lysosome, forming a phagolysosome, to allow the enzymes to digest the foreign object in isolation within the cell, without damaging the phagocyte itself (Alberts, 1983).

OXYGEN RADICAL PRODUCTION

The reduction of molecular oxygen results in the formation of the superoxide anion (O_2^-) at the expense of cytosolic nicotinamide adenine dinucleotide phosphate (NADPH)(equation 1). This reaction is mediated by the enzyme NADPH oxidase located within the plasma membrane (Babior, 1981; Henson, 1987):

$$2O_2 + 2e^- + NADPH \xrightarrow[\text{NADPH oxidase}]{} 2O_2^- + NADP^+ + H^+ \quad (1)$$

Superoxide anions are highly reactive and thus capable of reacting with biological substrates (Fridovich, 1986). As the radical can act as both an oxidant as well as a reductant (accept or donate an electron), two of these radicals preferentially interact, spontaneously or aided by the enzyme superoxide dismutase (SOD), to form a molecule of hydrogen peroxide (H_2O_2):

$$2O_2^- + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2 \quad (2)$$

However, O_2^- can not be tolerated by living systems since, together with the less reactive but more persistent H_2O_2, it can undergo the Haber-Weiss (Halliwell, 1989) reaction to generate the highly reactive but short lived hydroxyl radical (HO·):

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + HO\cdot \quad (3)$$

This reaction is kinetically very slow but may occur *in vivo* in the presence of a ferrous salt, as described by Fenton as in equations 4 and 5 (Tauber, 1985):

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (5)$$

An alternative to the hydroxyl radical, the ferryl radical ($FeOH^{3+}$) may be a product of the Fenton reaction (Halliwell, 1989):

$$Fe^{2+} + H_2O_2 \rightarrow FeOH^{3+} + OH^- \quad (6)$$

H_2O_2 can also oxidise the halide chlorine (Cl^-), mediated by the neutrophil enzyme myeloperoxidase (MPO), to form a powerful oxidant identified as hypochlorous acid (HOCl) as in equation 7 (Winterbourn, 1985):

$$H_2O_2 + \dot{C}l^- \xrightarrow[MPO]{} HOCl + H_2O \quad (7)$$

Both these long and short lived reactive oxygen intermediates are unstable and extremely reactive and thus important components of the oxygen-dependent killing of ingested microbes by phagocytic leucocytes. However, these highly reactive radicals may be released into the extracellular enviroment as waste products or accidentally. In such a situation when the local scavengers of reactive oxygen intermediates, the superoxide dismutases, catalases and peroxidases, are overwhelmed these radicals are capable of damaging host tissues.

PROTEOLYTIC ACTIVITY.

Neutrophil proteases are synthesised in the ribosomes of the rough endoplasmic reticulum and transported to the golgi complex where they are stored in the cytoplasmic vesicles. There are two types of cytoplasmic vesicles: the azurophilic or primary granules which contain acid hydrolases, neutral proteases, myeloperoxidase and lysosyme; and specific or secondary granules which contain lysosyme and lactoferrin. Also the plasma membrane contains alkaline phosphatase.

In some cases, e.g. during phagocytosis of a particle that is too big, membrane damage or cell necrosis may occur releasing the granular contents into the extracellular fluid. The serine proteinases (elastase and cathepsin G) and the metalloproteinases (collagenase and gelatinase) of the neutrophil can degrade elastin and collagen of the interstitium and basement membrane, and have the potential therefore to also degrade host tisssues. Normally the presence locally of an abundance of antiproteases would protect host tissue from degradation by any proteases which are released. However, reactive oxidants generated by neutrophils are capable of inactivating the proteinase inhibitors locally around the inflammatory neutrophil (Ossanna, 1986). This has inspired the hypothesis that the presence of activated neutrophils at sites of inflammation may tip the delicate balance between local tissue destruction and protection, and result in damage of host tissue (Weiss, 1989).

1.3 HAEMORHEOLOGY

Rheology is the study of the flow and deformation properties of a material or system of materials. Blood flow and deformation - haemorheology - is determined by the viscosity of plasma and the intracellular fluids, and viscoelastic properties of the membranes of blood cells (Lowe, 1987; Skalak, 1989).

1.3.1 THE PHYSICAL CHARACTERISTICS OF BLOOD CELLS

Erythrocytes are the cell type of greatest abundance, constituting 40-45% of the blood by volume. Human erythrocytes have a surface area (140 μm^2) which is 44% in excess of the surface area required to cover a sphere of equal volume (90 μm^2)(Chien, 1984). The biconcave discoid shape of erythrocytes is important for transit in narrow vessels, such as the capillaries, as it leads to preferential axial positioning compared with spherical shaped objects such as the leucocytes (Schmid-Schonbein, 1980a). As well as the geometry of the cell, the viscoelastic properties of the erythrocyte membrane may determine erythrocyte deformability (Schmid-Schonbein, 1981). The membrane is influenced by both the lipid composition of the

membrane (Garnier, 1985) and the subcortical cytoskeleton (Chabanel, 1983). However, the internal viscosity of the erythrocytes is probably most influential for cell deformability (Schmid-Schonbein, 1981). The intracellular contents of the erythrocyte consists of a haemoglobin solution, which is a Newtonian fluid, and devoid of a nucleus. Erythrocyte deformability decreases with increasing haemoglobin concentration (Reinhart, 1985).

The heterogenous leucocyte population (neutrophils, eosinophils, lymphocytes and monocytes) constitute the next largest cell population in the blood, but make up less than 1% of the total volume. Although of a similar diameter (6.2 μm - 7.5 μm) to the erythrocyte, being spherical they have a larger volume (190 μm^3 - 230 μm^3)(Beyers, 1989; Schmid-Schonbein, 1980b). Leucocytes have a viscoelastic interior (Schmid-Schonbein, 1981) which is approximately 1000 fold more viscous than that of the erythrocyte (Cokelet, 1968). This is due mainly to the large, non-deforming nucleus and granular cytoplasm present in leucocytes, and an active cytoskeleton which affects both the cell interior and the plasma membrane. Leucocytes have a highly ruffled plasma membrane producing an excess surface area of 84% - 137% required to cover a smooth sphere of equivalent diameter (Schmid-Schonbein, 1980b). The membrane elasticity of the neutrophil was found, by aspirating part of the cell into micropipettes, to be four fold less than that of the erythrocyte membrane (Chien, 1984). Hence, the rheological properties of leucocytes make them several orders of magnitude stiffer than erythrocytes.

Human blood also contains platelets which are small (2-4 μm), round or oval discoid in shape and occupy less than 0.5% of the blood volume. Platelets therefore have a negligible effect on blood viscosity and hence rheology (Chien, 1984; Skalak, 1989). However, their role in blood clotting, by adherence to the endothelium and aggregation, may dramatically alter blood flow.

1.3.2 BLOOD FLOW

The flow of a liquid through a system of tubes results in a pressure drop along the way due to resistance offered by the vessels (Fahraeus, 1929). Poiseuille (1843) determined that the resistance offered by a tube to a traversing volume of simple (Newtonian) fluid is proportional to the length of the tube, and inversely as the fourth power of the diameter. Hence, a small change in the diameter of the tube must have a profound effect on the flow of a fluid. As well as the resistance of the tube, the flow of a fluid is subject to it's intrinsic resistance, i.e. the viscosity which arises

from internal friction. Flow is therefore also inversely proportional to the viscosity of a fluid and would likewise be greatly influenced by the vessel diameter.

Plasma and whole blood flowing in large vessels (>300 μm diameter) behave as Newtonian fluids, with a constant viscosity regardless of the shear conditions, to which Poiseuille's law can be applied (Fahraeus , 1929; Lowe, 1987). However, Fahraeus and Lindquist (1931) found that for vessels of smaller diameters (<300 μm), the viscosity of blood was far less than that predicted by Poiseuille's law. Fahraeus (1929) had earlier established that the haematocrit of blood in a tube decreased with decreasing tube diameter (<300 μm). Dilution of the erythrocyte suspension occurs as the cells flow in single file in the axial stream, forming a cell depleted stream along the vessel wall. As the cells in the axial stream have a greater velocity than the marginal stream, the apparent viscosity of the suspension decreases (the Fahraeus-Lindquist effect). Moreover, as the erythrocytes are passively deformed under high shear conditions to a convex front and concave rear shape in line with streamline flow (Dintenfass, 1962; Skalak, 1969), they flow as droplets in the axial stream and contribute to the Fahraeus-Lindquist effect by further reducing viscosity. Whole blood flowing in small vessels therefore does not obey Poiseuille's law.

Likewise, when the rate of flow in a tube is gradually increased to a point where laminar flow is replaced by turbulence, the conditions of Poiseuille's law no longer apply (Reynolds, 1883). Flow in the major vessels of the human circulation, although not turbulent except at branches and bends, does occur in waves and hence there are significant effects of inertia (mass opposing a change in motion). In the microvessels, however, there is no turbulence (Reed, 1985) and thus inertia has a negligible effect. The equations of motion applied to blood, under these conditions, can be reduced to Stokes' equations (Skalak, 1989).

The smallest tube in which blood viscosity was investigated by Fahraues and Lindquist (1931) was 50 μm in diameter. As the capillaries of the microcirculation have a calibre of around 5-6 μm, Dintenfass (1967) examined blood flow in capillaries of radii as small as 2.5 μm. His studies revealed an inverse effect of the Fahraeus-Lindquist phenomenon for blood (from humans, toads and amphiumas) flowing in tubes <5 μm in diameter. An increased viscosity was observed with decreasing diameter with the critical diameter being between 5 and 7 μm. Changes in the haematocrit of a suspension, by contrast, did not alter the Fahraeus-Lindquist phenomenon or the inverse phenomenon, even for haematocrits of 95% (Dintenfass, 1967).

1.3.3 LEUCOCYTE RHEOLOGY

It is apparent that the cellular constituents of blood influence blood flow. Nicoll and Webb (1946) were one of the first to observe the cessation of blood flow in the capillary segments of microvascular beds. It was only later this was attributed to leucocyte plugging in nutritive capillaries in man and animals (Bagge, 1976; Chien, 1983; Lien,, 1987b; Schmid-Schonbein, 1990). By studying the filterability of leucocyte and erythrocyte suspensions through 5 μm pore membranes at a constant flow, Chien and colleagues (1983) demonstrated that both erythrocytes and leucocytes have a concentration-dependent effect on blood filtration *in vitro*. By theoretical calculations they were able to establish the relative influence of each cell type. They found that approximately a 700 - 1000 times greater erythrocyte concentration was required to achieve the same effect as a leucocyte concentration. It is of interest to note that the ratio of leucocytes to erythrocytes in normal blood is 1:700. Moreover, as mentioned above, leucocytes have a cellular viscosity (Schmid-Schonbein, 1981) approximately 1000 times greater than that of erythrocytes (Chien, 1981; Cokelet, 1968), hence both of these cells are equally likely to affect blood flow *in vivo*. However, these data also suggest that leucocytes have a negligible effect on blood viscosity. Although this is true for bulk fluids, because of their rheological properties, leucocytes have a marked effect on the microrheology of blood.

The dimensions of the microvasculature, relative to that of the erythrocytes and leucocytes, imposes a considerable restraint on neutrophil passage particularly within the pulmonary capillaries. In the smaller capillaries, the larger blood cells must deform to diameters as small as one half of their normal undeformed diameter. The disparity between the sizes of the circulating leucocytes (6-8 μm)(Schmid-Schonbein, 1980a) and the pulmonary capillaries (mean 5 μm)(Weibel, 1963) requires that one or other must deform during capillary transit of the cells. Although changes in capillary diameter, as in the zones of West (1964) described above, would affect leucocyte transit, an increase in capillary diameter to accommodate the larger leucocytes and erythrocytes during transit has not been reported.

The important influence of leucocytes on blood flow has been established using *intra vital* microscopy (Bagge, 1976; Chien, 1985; Gaehtgens, 1982; Lien, 1987b; Schmid-Schonbein 1980b; Wagner, 1982) and *in vitro* models (Bagge, 1976; Chien, 1983;

Daily, 1984; Downey, 1988; Frank, 1990b; Lennie, 1987; Nanmark, 1989; Nash, 1990). Leucocytes have been observed, in such studies, to reduce blood flow in narrow capillaries of diameters similar or smaller than the cells themselves. Schmid-Schonbein and colleagues (1980b) demonstrated, *in vitro* using spherical and disc shaped objects to mimic the blood cells, that for narrow capillaries and low flow conditions a "train" of erythrocytes piled up behind the slower flowing leucocytes, and a plasma gap, depleted of erythrocytes, formed downstream. The authors found that the larger volume and stiffness of leucocytes, compared with that of erythrocytes, caused a reduction in the axial velocity of leucocytes. The axial velocity of the spheres or leucocytes was further reduced when displaced towards the vessel wall by the faster flowing discs or erythrocytes (Schmid-Schonbein, 1980b). Although both cells being of equal size are in contact with vessel walls of the smaller capillaries, only the leucocytes are capable of adhering which can lead to partial obstruction of the vessel lumen and an increase in the flow resistance of the vessel (Schmid-Schonbein, 1980b). Furthermore, Gaehtgens, Pries and Nobis (1984) observed that leucocytes downstream from a stenosis in a capillary tube had a slower velocity than leucocytes upstream from the stenosis, which they attributed to the deformed shape of the cell (after squeezing through the capillary stenosis) as it took time to recover its undeformed shape.

In converging vessels, such as capillaries merging into post-capillary venules, when the tube dimension is increased sufficiently the faster flowing erythrocytes can pass the leucocytes, displacing them towards the venule wall. Similarly, at a sudden step increase in vessel lumen, the biconcave discoid shape of the erythrocyte leads to its positioning in the centre of the vessel lumen, where its increased velocity causes displacement of the leucocytes (Schmid-Schonbein, 1980b). Moreover, with enough displacement, contact with the vessel wall and a rolling adhesive interaction of leucocytes with the endothelium can occur. This location of leucocytes in the marginal regions of the postcapillary vessels has been termed "margination".

Likewise for larger vessels, Nobis and colleagues (1985) demonstrated *in vitro* that the mechanism for leucocyte margination was by displacement of leucocytes, and prevention of their re-entry into the axial stream by the presence of large numbers of erythrocytes or aggregates formed under slow flow conditions. *In vivo* studies in the systemic circulation, such as the rabbit ear, hamster cheekpouch, cat, mouse or rabbit mesentery, and also in the human finger nailfold have confirmed these *in vitro* observations (Atherton, 1973; Bagge, 1976; Chien, 1985; Schmid-Schonbein, 1980c) .

More pertinent to the work in this thesis, *intra vital* studies have also been performed in the lungs of dogs. In their studies, Lien and associates (1987b; 1990), observed

that fluorescently labelled neutrophils were delayed in the pulmonary microvasculature compared with plasma transit times (determined using fluorescent dextran) under both normal and local inflammatory conditions. This was observed to occur almost exclusively in the capillaries as opposed to the postcapillary venules (Lien, 1987b), and at discrete sites (Lien, 1990). The restriction of neutrophils at only a few of such sites greatly impaired their pulmonary transit (Lien, 1990). The slower transit of neutrophils in the capillaries may be due to the size discrepancy between the leucocyte and capillary dimensions and/or adherence to endothelium. Both of which would be enhanced by the slower blood flow in the capillaries.

The terminology used in the literature (margination, retention, and sequestration) to describe leucocyte kinetics relative to erythrocytes in microcirculatory beds can be confusing. The margination of cells, as detailed above, refers to non-circulating leucocytes located along the vessel wall, which occurs predominantly in postcapillary venules at least in the systemic circulation (Schmid-Schonbein, 1975; Schmid-Schonbein, 1980b). The 'retention' of neutrophils in tissues should be used to describe only the number of cells retained as a percentage of cells delivered to the tissue, and therefore needs knowledge of all cells delivered. Whereas the term 'sequestration' encompasses all non-circulating cells, i.e. the marginated neutrophils as well as those trapped or moving slowly due to constrictions of the vessel lumen and those adherent to the endothelium.

1.4 LEUCOCYTE NUMBERS AND LUNG DISTRIBUTION.

1.4.1 EARLY STUDIES

The observation of changes in the number of white cells in the circulation first received attention in the mid 19th Century, which was linked with inflammatory diseases several decades later (Cohnheim, 1867; van Limbeck, 1890).

As early as 1867, Cohnheim (1867) observed that a small number of leucocytes assumed a marginal position in the blood stream at sites of inflammation. The author attributed this to a reduction in blood velocity. Cohnstein and Zuntz (1888) suggested this margination of leucocytes was the cause of their accumulation in some vascular regions and disappearance from other parts of the vascular system. In agreement with these observations, a decade later Rieder (1892) investigated the distribution of white cells in the vascular system of rabbits and found an increase in the number of white blood cells in the peripheral veins compared with the large

central veins. He attributed this phenomenon to the slow blood flow in the smaller veins and adhesion of white cell to endothelial cells.

Workers in Germany (Goldscheider, 1894) and England (Bruce, 1894) simultaneously provided evidence to suggest a marginating pool of leucocytes also existed within the vasculature of the lungs. The study of white cell distribution was further advanced when investigators realised that both a leucocytosis and a leucopenia, i.e. an increase in number or loss of leucocytes from the circulation respectively, could be induced experimentally (reviewed by Vejlens (1938)). Although the initial explanation for the decrease in leucocytes in the blood (leucopenia) was thought to be as a result of destruction of these cells (Lowit, 1885), it was later realised that changes in leucocyte numbers in the circulation could be due to changes in distribution in the vascular system (Schulz, 1893). Goldschieder and Jacob (1894) found that intravenous injection of bacteria into rabbits resulted rapidly in a leucopenia, measured by sampling blood from the rabbits ear, which was followed by a leucocytosis a few hours later. Examination of various organs during a leucopenic state revealed increased numbers of white cells in all organs with the greatest increase in the lungs. Moreover, during the subsequent leucocytosis, Goldscheider and Jacob (1894) noted that the number of leucocytes in the lungs increased even further which must have been caused by an increase in absolute number. Around the same time, Bruce (1894) also observed that leucocytes 'withdrew' from the circulation under normal conditions, to sequester in the vasculature of organs such as the lungs, liver and spleen. Likewise, by studying the histology of these organs Andrewes (1910) confirmed an increase in neutrophil numbers during a leucopenia, but only in the lungs.

1.4.2 CARDIAC CATHETERISATION STUDIES

The introduction of cardiac catheterisation allowed Bierman and colleagues (1951) to measure the number of leucocytes entering and leaving the lungs. They concluded that the sequestration of leucocytes in the pulmonary circulation was a normal physiological phenomenon. The same investigators proceeded to demonstrate the influence of changes in haemodynamics and the effect of respiratory movements on leucocyte sequestration in the lungs (Bierman, 1952a). Using epinephrine to increase, or histamine to decrease blood flow they were able to demonstrate a reduced or an enhanced leucocyte sequestration respectively (Bierman, 1953; 1952b; 1951). However, a decrease in neutrophil adhesivity may have contributed to the release of sequestered neutrophils from the lungs as catecholamines are reported to activate the

β-adrenergic receptors on neutrophils which decreases their adherence to endothelium *in vitro* (Ahlborg, 1970; Boxer, 1980; MacGregor, 1977).

Furthermore, using the valsalva manoever where the subject forcibly expires against a closed glottis, Bierman and associates (1952a) measured arterio-venous differences across the lungs, and even during quiet breathing, with inspiration the arterial count was observed to fall and the venous count to rise, with the converse occurring with expiration (Bierman, 1952a). More recently Markos and colleagues (1990) likewise demonstrated that a valsalva manoever against an occluded airway caused a delay in neutrophil lung transit and a reduction in the cardiac output. Similarly, Glazier and colleagues (1969), using isolated perfused dog lungs, investigated the effect of changing alveolar and vascular pressures in the lungs on capillary dimensions and recruitment of new vessels. They found that increasing the alveolar pressure, which could further reduce the pulmonary capillary diameter, reduced the number of erythrocytes per septum, whereas increasing the capillary pressure had the opposite effect. Although Glazier et al (1969) made no comment about leucocyte numbers, pressure-induced changes in the number of erythrocytes could also reflect leucocyte entrapment. Taken together these data suggest that the observed neutrophil accumulation and reduced blood flow was due to compression of lung capillaries. This would enhance the size discrepancy between the capillary and neutrophil diameters, temporarily trapping a proportion of the larger neutrophils, with a higher concentration of cells leaving the lungs for a brief period thereafter. Moreover, Perlo and associates (1975) demonstrated that within the lungs the range of hydrostatic pressures acting on capillaries, which can be separated into the three zones of West described above, influenced the retention of neutrophils relative to that of erythrocytes. The distensibility of the lung vessels at the base of the lungs (Zone III), alters the hydrostatic pressure, reducing the resistance which would allow leucocytes to transit the capillaries more easily. However, the recruitment of new vessels, which occurs in zone II (middle) of the lung, could enhance leucocyte sequestration by enlarging the capillary bed (Glazier, 1969).

1.4.3 RADIO-ISOTOPE STUDIES

The introduction of radio-isotope cell labelling techniques and non-invasive external imaging have confirmed the early work. The use of radio-isotopes allowed quantification of the total blood leucocyte pool and the identification of two subpools. Using di-isopropylfluorophosphate (DFP^{32})-labelled leucocytes, Athens and coworkers (Athens, 1961) established that the total blood granulocyte pool in man was 0.65×10^9 cells.kg^{-1}, consisting of two subpools of equal size, the

circulating and the non-circulating marginated or sequestered leucocyte pool. Similar data was reported by Cartwright (1964). More importantly, Athens and colleagues (1961) also noted that DFP^{32}-labelled leucocytes were lost to the marginated pool on administration of bacterial endotoxin, and could be returned into the circulating pool when subjects exercised or were given adrenalin, thereby demonstrating considerable flux between the two pools. This phenomenon has subsequently been observed by several others (Ahlborg, 1970; Doerschuk, 1988b; Muir, 1984; Peters, Allsop, 1992). The radio-isotope techniques have also confirmed that an inverse relationship exists between the size of the sequestered intrapulmonary pool and pulmonary blood flow (Doerschuk, 1988b & 1990a; Martin, 1987; Martin, 1982).

1.4.4 THE SITE OF NEUTROPHIL SEQUESTRATION IN THE LUNGS

Thus, from these studies it is apparent that the lungs are a major site of leucocyte sequestration. In the systemic circulation neutrophils have been observed to marginate principally in post-capillary venules. By contrast, several studies have suggested that leucocytes are predominantly retained within the pulmonary circulation in the alveolar capillary bed rather than the post capillary venules (Braide, 1989; Doerschuk, 1990a; Hogg, 1987 &1988; Lien, 1987b). Support for this comes from morphological studies which have demonstrated a greater ratio of leucocytes to erythrocytes in lung capillaries in dog lungs. Perlo and coworkers (1975) observed a predominance of lymphocytes located in the capillaries of rapidly frozen dog lungs, although the arterioles and venules were not specifically examined in their study. Whereas Hogg and co-workers (1988), also studying the dog lung, found mainly sequestered neutrophils with the greatest number within the capillaries. Likewise, in rabbit lungs, neutrophils were located mainly within the capillaries (Doerschuk, 1987). Probably, the most convincing evidence demonstrating the sequestration of neutrophils in capillaries has been provided by direct visualisation of circulating cells, using intravital microscopy. Lien and associates (1987b) observed that neutrophils were delayed for longer than erythrocytes (Wagner, 1982) within the pulmonary capillary network, with little evidence of any sequestration occurring in the arterioles or venules. Moreover, Downey and associates (1993), using morphometric techniques, observed neutrophil migration occurred from the capillaries of the lung. This strategic site of neutrophil sequestration in close proximity to the external enviroment, places the cell in an ideal position for reacting to local extravascular chemotactic stimuli, to carry out its defensive phagocytic function against inhaled pathogens or irritants. Margination in the post-capillary

venules, on the other hand, would necessitate cell migration over a greater distance before reaching the site of injury.

1.5 NEUTROPHILS AND LUNG INJURY

1.5.1 THE INFLAMMATORY PROCESS

As an initial response to injury or infection, a local inflammatory reaction is one of the most important defence systems of the body. Inflammation is usually classified in terms of an acute or a chronic reaction. An acute inflammatory reaction is characterised by rapid occurrence and resolution, whereas chronic inflammation persists (Gallin, 1988).

In response to injury or infection, capillaries in the area contract briefly and thereafter dilate, causing endothelial cells to swell and the vessels to leak (Gallin, 1988). The inflammatory exudate which develops contains lymph fluid and white blood cells which migrate by chemotaxis to the site of injury. Polymorphonuclear leucocytes are the first of the white cells to appear at the site of tissue damage or infection, followed by lymphocytes and monocytes several hours later (Gallin, 1988). Blood monocytes at the site of injury mature into macrophages which proliferate and, together with proliferating lymphocytes (forming granulomas) and plasma cells, dominate the inflammatory site.

As the lung has a large internal surface open to air, it is particularly vulnerable to infection and injury which could initiate an inflammatory reaction. However, the lungs contain a comprehensive non-immune and immune defense system. The ciliated airway epithelia "sweep" foreign particles which settle in the surface of mucus secreted by goblet cells, up into the pharynx to be swallowed. At the alveolar surface, the phagocytic (macrophages and leucocytes) and immune cells (lymphocytes, plasma cells) destroy or remove any undesired material. However, malfunction of this beneficial process could instead be injurious to host tissue.

1.5.2 LUNG INJURY IN MAN

Excessive neutrophil sequestration may not always result in lung injury. Other factors, such as cell activation and the degree of contact between neutrophils and the vascular endothelium also contribute. However, impaired neutrophil deformability has been linked with tissue injury in man, such as during the development and progression of ischemia in the heart (Dahlgren, 1984; Nash, 1989), brain (Ciuffetti, 1989a) and lower limb (Ciuffetti, 1989b; Nash, 1988c; Neumann, 1990).

Evidence for neutrophils mediating lung injury has come from observations made in clinical conditions. Craddock and associates (1977) initially observed that haemodialysis activated the complement cascade which caused leucocytes to accumulate in the lungs, leading to tissue damage and pulmonary dysfunction. This initiated the study of neutrophils in a variety of disorders characterised by lung inflammation, such as sepsis (Hangen, 1990), adult respiratory distress syndrome (ARDS)(Weiland, 1986), chronic obstructive pulmonary disease (COPD)(Selby, 1991a), interstitial lung diseases (ILD)(Hunninghake, 1981 & 1981b), pancreatitis, major trauma and other severe insults (Pepe, 1982). It has been suggested that the enhanced number of leucocytes in the lung capillaries causes endothelial damage by releasing toxic metabolites such as oxygen free radicals and proteolytic enzymes (Donnelly, 1992; Tagan, 1991). Increased numbers of neutrophils have been found in lavage fluids from patients with destructive lung diseases, including ARDS (Fowler, 1982; Lee, 1981; Weiland, 1986), ILD (Hunninghake, 1981b; Reynolds, 1977; Roberts, 1993) and cystic fibrosis (Fick, 1984). Moreover, neutrophil products capable of mediating lung damage have been detected in such patients (Burnett, 1987; Fowler, 1982; Lee, 1981; Stockley 1982; Weiland, 1986). Indeed, in inflammatory conditions such as ARDS and COPD, neutrophils have been associated with impaired gas-exchange (Richards, 1989; Weiland, 1986) and increased lung epithelial permeability (Tate 1983; Weiland, 1986).

1.5.3 ANIMAL MODELS OF LUNG INJURY

Manipulations in animal models have confirmed that neutrophils are important mediators of lung injury. Acute lung injury followed the administration of activators such as phorbol esters (Shasby, 1982), chemotactic factors (Lien, 1987a; Tanaka, 1992), complement factors (Lien, 1991), or bacterial toxins (Brigham, 1974; Haslett, 1987; Worthen, 1987a). Haslett and associates (1987) observed an extensive pulmonary sequestration of neutrophils in a rabbit model following intravascular administration of endotoxic lipopolysaccharide (LPS) which confirms the earlier work (Goldscheider 1894; Vejlens, 1938) of a leucopenia following the injection of various gram negative bacteria into animals, with neutrophils localising in the lungs. LPS is the active moiety released from bacterial cell walls which initiates both the classic and alternative complement cascades in plasma (Morrison, 1977). It is, therefore, not surprising that infusion of zymosan activated plasma into rabbits also induced a marked leucocyte accumulation in the lung microvasculature (Doerschuk, 1989), associated with both a mild epithelial and endothelial injury (Gie, 1991).

Moreover, instillation of the complement factor C_{5a}, a strong neutrophil chemoattractant, into hamster or rabbit airway resulted in an intense inflammatory response characterised by neutrophil accumulation (Desai, 1979) and resulting in damage to the pulmonary parenchyma (Desai, 1979; Hellewell, Henson, 1991; Shaw, 1980).

Gram-negative sepsis and endotoxin may also initiate inflammatory stimuli other than the complement cascades, such as the cytokines (Stevens, 1988), causing neutrophils to accumulate in the lungs. However, as LPS-pretreated neutrophils also sequestered in the lungs of rabbits, it suggests a complement-independent mechanism is involved (Haslett, 1987).

Moreover, complement activation was unable to simulate the potency of LPS (Meyrick, 1983 & 1984). A possible explanation may be that LPS can directly injure lung endothelium (Gaynor, 1973; Meyrick, 1983). More likely, as suggested by Haslett, the major destructive effect of LPS may be by "priming" neutrophils (Haslett, 1987) so that upon stimulation their generation of oxygen radicals (Guthrie, 1984; Haslett, 1985) and proteases (Haslett, 1985) is enhanced. This is supported by the enhanced sequestration of neutrophils, resulting in lung endothelial injury and increased vascular permeability, following the intravascular administration of a combination of small amounts of LPS with complement fragments or synthetic chemotactic factors, but not by either agent alone (Worthen, 1987a). This is of particular relevance with regard to the lung disorder, ARDS which occurs by a combination of oxygen toxicity and gram negative sepsis (Rinaldo, 1988). Oxygen toxicity alone was observed, by Rinaldo and co-workers (1988), to alter neutrophil kinetics in the lung, but inflammation did not develop.

Moreover, inhibition of the neutrophil respiratory burst (Carey, 1990) and inhibition of leucocyte adhesion (Ismail, 1987; Walsh, 1991) significantly attenuated lung injury in animal models implicating leucocytes as mediators. Furthermore, it was shown that leucocyte depletion prevented lung injury (Gie, 1991; Heflin, 1981; Johnson, 1980; Stevens, 1988).

Clearly, there is much evidence from animal studies to implicate the involvement of leucocytes in the initiation and the development of lung injury.

1.5.4 EVIDENCE AGAINST NEUTROPHIL INVOLVEMENT IN LUNG INJURY

Although not as extensive, there is also evidence to suggest that the neutrophil is not an essential cellular mediator of tissue damage. In contrast to the studies mentioned above, animal studies and isolated lung preparations show prior depletion of leucocytes abolished the lung injury which developed in the presence of leucocytes.

For example, experiments in neutropenic animals have shown that the effects of hyperoxia or endotoxin-induced lung injury were neutrophil-independent (Dener, 1973; Schwartz, 1983; Shasby, 1982b). In man, Rinaldo (1985) observed continuing deterioration in lung mechanics in leukemic patients despite resolution of chemotherapy-induced neutropenia. Moreover, Maunder and associates (1986) found patients severely neutropenic due to chemotherapy were still able to develop ARDS. However, this was contradicted by an observation made by Weiland and colleagues (1986) that during the two years of their study no neutropenic patients developed ARDS.

1.6 EMPHYSEMA

1.6.1 DEFINITION OF EMPHYSEMA

Emphysema is a chronic disorder of the lower respiratory tract. The word emphysema, derived from the Greek, means inflation, but the disorder is also characterised by destruction of alveolar walls. Laennec (1821) originally described the clinical and pathological features of emphysema, and his description dominated the field until reviewed by a group of British physicians in 1959 (Ciba Foundation Guest Symposium, 1959) and subsequently by the World Health Organisation (WHO, 1961) and American Thoracic Society (ATS, 1962) to be redefined as:

> 'a condition of the lung characterised by abnormal, permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by the destruction of their walls, and without obvious fibrosis' (Snider, 1985).

The lungs' tissue matrix provides the architectural support for the alveolar walls and modulates the mechanical properties of the lung parenchyma during respiration. The major component of this tissue matrix is the elastic fibre, elastin. Elastin fibres can stretch to several times their length, and rapidly return to their starting length when the tension is released. This is essential for the expansion and contraction of the lung (Weibel, 1984). Hence, destruction of elastin would be detrimental to the compliance of the lung.

Three major forms of emphysema exist, according to where the lesions develop within the lungs, i.e. centriacinar, panacinar or distal-acinar emphysema, where an acinar unit comprises airpaces arising from a single terminal bronchiole (Weibel, 1984).

Emphysema is prevalent in subjects deficient in the major proteinase inhibitor, α_1-protease inhibitor (α_1-PI, formerly known as α_1-antitrypsin) which normally inhibits elastase activity (Eriksson, 1989). However, the major cause of emphysema is the cigarette smoking habit (Auerbach, 1972). Indeed the extent of emphysema has been correlated with the degree of cigarette smoking (Auerbach, 1972; Petty, 1967).

Leopold and Gough (1957) first described the centrilobular form of emphysema, which is predominant in cigarette smokers (Thurlbeck, 1976). The lesions occur more frequently in the upper zones of the lung (Thurlbeck, 1963), and are most severe at the proximal sites in the acini (the respiratory bronchioles) with the parenchyma distal to the emphysematous lesions (the alveolar ducts, sacs and alveoli) being preserved (Dunnill, 1979). Hence, this form of emphysema is also referred to as centriacinar emphysema. Panacinar emphysema occurs in subjects deficient in α-$_1$PI, with most severe tissue degradation occurring at the base of the lungs (Laurell, 1963). As emphysema progresses it becomes increasingly difficult to distinguish between the centrilobular and panacinar forms of the disease. A third anatomic form of emphysema has also been identified and localised in the alveolar ducts and sacs and hence is known as distal-acinar emphysema.

1.6.2 THE DEVELOPMENT OF EMPHYSEMA

A PROTEASE/ANTIPROTEASE IMBALANCE AND ELASTIN DESTRUCTION

A popular hypothesis to explain the lung destruction observed in emphysema is that of a functional protease imbalance in the peripheral lung (Gadek, 1979; Janoff, 1983). This concept of a protease/antiprotease imbalance of emphysema evolved as a consequence of two important observations. Firstly, in 1963 Laurell and Eriksson (1963) observed that a deficiency of α_1-PI, the enzyme which normally inhibits elastase activity, was associated with the development of emphysema. Secondly, Gross and associates (1965) found that the intra-tracheal injection of papain, a proteolytic enzyme, into normal rats resulted in lung destruction similar to that of emphysema.

More recent studies have replicated and extended the work of Gross et al (1965) to establish that only enzymes capable of degrading elastin can induce emphysematous lesions (Janoff, 1977; Senior, 1977). Although a variety of neutral proteases have been evaluated as potential candidates in causing the alveolar wall destruction (reviewed by Niewoehner (1988)), most attention has focused on elastase. Janoff and Scherer (1968) first demonstrated elastase activity in the human neutrophil granules. Elastase is capable of cleaving the major connective tissue proteins that form the connective tissue matrix of the alveolar walls, particularly elastin (Janoff,

1985). Elastase has also been implicated in smokers emphysema by the detection of increased levels and/or functional activity of elastase in bronchoalveolar lavage (BAL) after a period of intense smoking (Fera, 1986; Janoff, 1983). Several years earlier, Galdston and colleagues (Galdston, 1977) reported increased levels of elastase in neutrophils in the peripheral blood of smokers. Moreover, Abboud and associates (Abboud, 1986) found an increase in the levels of immunoreactive elastase in plasma during acute smoking which was confirmed in arterial blood by MacNee and colleagues (1989a). Furthermore, Damiano and colleagues (1986), using immunostaining, demonstrated elastin associated neutrophil elastase in emphysema patients, although activity was not assessed. This study could not be replicated by Fox et al (1988), however, both Kukich (1985) and Weitz (1987) were able to demonstrate increased levels of neutrophil elastase-induced elastin degradation products in the blood of smokers. Furthermore, to add to the neutrophil protease burden, lung macrophages can be activated or damaged to release cathepsin B and L (Johnson, 1978), and possibly neutrophil elastase previously taken up by the macrophage (Campbell, 1979; Janoff, 1985).

ANTIPROTEASE ACTIVITY IN THE LUNGS

In the normal human lung mainly α_1-PI and α_2-macroglobulin (Henson, 1988; Ogushi, 1991) and low molecular weight inhibitors such as antileukoproteinase (Bruch, 1986) protect the connective tissue components of the alveolar walls from destruction by elastase. The inhibitor α_1-PI, produced by hepatocytes and mononuclear leucocytes, has a high association rate constant for neutrophil elastase. Moreover, whereas the low molecular weight inhibitors behave as competitive inhibitors (Afford, 1986), α_1-PI is an irreversible inhibitor of the enzyme (Beatty, 1984). Thus α_1-PI appears to be the major protease inhibitor preventing elastase from attacking alveolar walls (Gadek, 1981; Ohlsson, 1971). However, the antiprotease activity of low molecular weight inhibitors can not be ignored. Indeed, several studies propose α_1-PI accounts for only 10% of the antielastase activity in lavage fluid (Boudier, 1983; Stockley, 1984). The low molecular weight inhibitors have the advantage over large molecular weight inhibitors such as α_1-PI in that they have the ability to penetrate the pericellular space between an adhered leucocyte and the connective tissue. Morrison (1987) proposed the concept that the inhibitors in the lower lung interact as a chain of inhibitors. A break in the chain, such as inactivation of α_1-PI, could lead to diminished antiprotease activity and tissue injury.

In chronic smokers the functional activity of α_1-PI in the lower respiratory tract was reported, by Gadek and colleagues (1979), to be reduced compared with non-smokers. Similarly, Janoff's group (Carp, 1982) reported a two fold reduction in the functional activity of α_1-PI in BAL from smokers compared to non-smokers. More recently, Ogushi and coworkers (1991) demonstrated that the association rate constant of α_1-PI purified from lavage fluid for neutrophil elastase was 20% less for smokers than non-smokers. Taken together these data suggest that α_1-PI present in the lower respiratory tract of smokers has a reduced inhibitory capacity for elastase.

INACTIVATION OF α_1-PROTEINASE INHIBITOR

The active site of α_1-PI contains a methione - serine bond (Johnson, 1978). Integrity of this bond appears to be essential for its function as oxidation of the methione residue at the active site reduced it's inhibitory activity (Carp, 1978; Johnson, 1979). Also, the association rate of the inhibitor with neutrophil elastase was reduced by a factor of 2000 following methionine oxidation (Beatty, 1980). Analysis of bronchoalveolar lavage (BAL) obtained from smokers revealed the presence of methionine residues in the sulfoxide form, suggesting exposure of methionine to an oxidant burden (Johnson, 1979). Further evidence to suggest oxidation of the inhibitor was supplied by Janoff and colleagues (1979b). These authors measured a decrease in the elastase inhibitory capacity per milligram of α_1-PI in lavage fluid from rats exposed to three or six puffs of cigarette smoke. Moreover, ozone-tolerant rats exposed to smoke had unaltered levels of α_1-PI activity in their lungs, unlike control rats exposed to smoke alone (Janoff, 1979b).

Oxidative inactivation of α_1-PI can occur by several pathways. The oxidants in cigarette smoke, both the particulate and gas phase, are capable of oxidising α_1-PI in the alveolar space, thereby inactivating the inhibitor (Carp, 1978). Furthermore, nitrogen oxides (nitric oxide (NO) and its derivative nitrogen dioxide (NO_2)) present during the burning of a cigarette, as well as their own free radical activity, may interact with phagocyte derived oxidants to generate further potent oxygen radicals such as the hydroxyl radical (Dooley, 1982; Halliwell, 1989).

Inactivation of α_1-PI may also occur by oxygen radicals produced by smoke-stimulated phagocytes (Johnson, 1979). Carp and Janoff (1979) found PMA stimulated mononuclear and polymorphonuclear leucocytes were capable of inactivating serum α_1-PI, and Clark and colleagues (1981) demonstrated oxidation of α_1-PI by the phagocyte myeloperoxidase (MPO) - system which gives rise to powerful oxidising agents such as hypochlorous acid. Moreover, Janoff and colleagues (1979b) found the elastase inhibitory capacity per milligram of α_1-PI in

smokers serum was reduced by 20% following the acute smoking of 1 cigarette. In contrast, a recent study found α_1-PI's ability to inhibit neutrophil elastase was unchanged in smoker's plasma (Ogushi, 1991). These discordant reports likely reflect a difference between an acute and chronic effect of smoke exposure on α_1-PI levels in plasma. Phagocyte-derived oxidants may, therefore, play a role in the inactivation of α_1-PI in the blood and the airways (Hoidal, 1981).

There is also the potential, which must be considered, that elastase can be inactivated by oxidants derived from cigarette smoke and leucocytes. Both Ohlsson (1980) and Ejiofor (1981) and their coworkers demonstrated partial inactivation of elastase by co-incubation with cigarette smoke condensate (CSC). However, neither study compared the effect of CSC on α_1-PI. Janoff and associates (1982) completed the full experiment to find that the elastase inhibitory capacity of serum was more susceptible to inactivation by aqueous cigarette smoke than neutrophil elastase.

IMPAIRED ELASTIN SYNTHESIS

To compound the situation, fresh cigarette smoke was found to inhibit the resynthesis of elastin, both *in vitro* and *in vivo*. Laurent (1982) and colleagues reported a 80 - 90% inhibition in elastin synthesis as assessed by reduced desmosine synthesis, the major cross-linking amino acid in elastin, in the presence of aqueous extracts of whole cigarette smoke. The vapour phase component of cigarette smoke in solution was also inhibitory in a dose dependent manner (Laurent, 1983). Moreover, Osman and associates (1982) observed a reduction in the rate of elastin resynthesis in emphysematous hamsters, induced by intratracheal installation of pancreatic elastase and daily exposure to cigarette smoke when compared with elastase-instilled hamsters not exposed to smoke.

Considerable evidence, therefore, suggests that cigarette smoke-related emphysema occurs by degradation of the elastin component of lung tissue, due to an increased elastase burden in the lung, which may be compounded by a reduction in elastin resynthesis. The blood and alveolar leucocytes have been implicated as important sources of elastolytic enzymes as chronic cigarette smoking is associated with an increased number of leucocytes in the peripheral circulation (Corre, 1985), with enhanced sequestration in the pulmonary parenchyma (Hunninghake, 1983) as well as their presence in increased numbers in the lower respiratory tract (Gadek,, 1979; Hunninghake, 1983). Furthermore, an increase in the spontaneous (Hoidal, 1982) and stimulated (Ludwig, 1982) superoxide anion production was reported for

neutrophils obtained from smokers compared with non-smokers. The neutrophil's ability to generate reactive oxidants would add to the oxidant burden of cigarette smoke itself.

Considering the destructive potential of phagocytes, Hogg (1987) proposed a plausible reason for the location of emphysematous lesions in smokers. Blood to the alveoli is supplied by the pulmonary circulation, whereas the bronchioles are supplied by the bronchial circulation. The blood supply enters at the acinus, dividing into arterioles then capillaries, and draining into the veins at the periphery of the acinus. Hence, the first contact neutrophils have with inhaled cigarette smoke, and thus their possible activation, would be in the centre of the acinus where emphysema is predominant in smokers. Moreover, as neutrophil transit is slower in the upper than in the lower lung regions (Martin, 1987), there is greater potential for neutrophil activation and the development of a local proteolytic imbalance.
Hogg (1987) also suggested a mechanism for the tissue destruction observed with α-$_1$PI deficiency. As neutrophil transit in the lower lung region is faster (Martin, 1987), a greater number of leucocytes pass through these lung capillaries. A greater number could therefore be retained with the potential of releasing their toxic complement.

In view of Hogg's hypothesis for the localisation of emphysematous lesions, and the evidence implicating leucocytes as mediators in the development of this disease, the study by MacNee and associates (1989d) demonstrating enhanced lung sequestration of neutrophils <u>during</u> smoking, is a major step forward in understanding the pathogenesis of emphysema and other smoking-related diseases. Establishing the mechanism of this smoke-induced sequestration could be an important link in the pathogenesis of these diseases.

1.7 CIGARETTE SMOKING

1.7.1 THE INTRODUCTION OF CIGARETTE SMOKING

Tobacco has been used in various forms for centuries. Tobacco cultivation began in Virginia, USA and was brought to Europe in the middle of the 16th Century. Tobacco has since played an important role in national and international economics, particularly with the beginning of the cigarette industry in 1918 (Heseltine, 1987).
In England, tobacco consumption increased throughout the seventeenth century, mainly in the form of pipe smoking. Smokeless forms of tobacco have also been used since the sixteenth century, such as chewing tobacco and snuff which is used by

placing between the lips and gums of the mouth or by nasal sniffing. Cigars were introduced to Britain at the beginning of the nineteenth century, but their popularity was never as great as in Europe. Cigarettes were first made in Brazil, in the eighteenth century, and brought to Britain by troops returning from Crimean war (Royal College of Physicians, 1971). Cigarettes are the most common form of tobacco use in the developed world today (Beese, 1972; Todd, 1972; Wald, 1988).

1.7.2 THE DETRIMENTAL EFFECTS OF CIGARETTE SMOKING

Initially the adverse effects of smoking were not recognised. Smoking was even recommended, by Jean Nicot the French Ambassador to Lisbon after whom nicotine was named, as being of medicinal value (Heseltine, 1987). For many years cigarette smoking was proposed as a slimming aid and, by the power of advertising, associated with fitness and style. An early report by Pearl (1938), that heavy smokers had a higher death rate than non-smokers was ignored. Even a decade later the Journal of the American Medical Association still observed that "smoking in moderation does not appreciably shorten life" (Am Med Assoc, 1948).

The risks associated with cigarette smoking were eventually acknowledged in the 1950's following publications of a causal relationship between cigarette smoking and lung cancer (Minister of Health, 1957). Subsequently, large surveys in various countries have established an increased risk of premature death in smokers compared with non-smokers (Darby, 1989; Doll, 1964; Kiryluk, 1989; Minister of Health, 1957; Todd, 1972; Uitenbroek, 1993; US Government, 1967; Wald, Kiryluk, 1988; Williams, 1991). The risk of a person dying in the course of a year was related to increasing number of cigarettes smoked, by inhalation of the smoke, and the earlier in life that smoking was undertaken. Moreover, the shorter life expectancy for smokers increases with advancing age (Heseltine, 1987; Royal Coll Phys, 1971; Wald, 1988; Williams, 1991). However, following cessation of smoking these risks steadily decline (Heseltine, 1987; Royal Coll Phys, 1971; Wald, 1988).

Not only does cigarette smoking shorten life, it also causes ill health. This is evident from the large number of working days lost (Higgins, 1991) and ultimately the poor quality of life experienced by some smokers, such as the years of breathlessness suffered by patients with emphysema and chronic bronchitic. Chronic Obstructive Pulmonary Disease (COPD: largely Chronic Bronchitis and Emphysema) is as specifically related to smoking as lung cancer (Auerbach, 1972; Darby, 1989; Doll, 1964; Niewoehner, 1988; Petty, 1967; Tobacco Research Council, 1969). A decrease in lung function in COPD patients has been correlated with the number of cigarettes smoked (Richards, 1989).

Airflow obstruction has been attributed to abnormalities in the lung parenchyma (emphysema)(Auerbach, 1972) and lesions in the airways including fibrosis, smooth muscle hypertrophy and enlargement and overactivity of the mucus glands (chronic bronchitis)(Niewoehner, 1988; Schultz, 1991). Destruction of the lung in emphysema impairs oxygen uptake by the blood as the surface area for gas exchange in the lung is reduced. The excessive mucus production can also hinder oxygen diffusion across the respiratory epithelium as well as interfering with the defence mechanism of the lungs by entrapping particles/microorganisms preventing their removal by ciliated lung cells. Moreover, the function of these cells may be directly affected as cigarette smoke is reported to be cilia-static (Tobacco Research Council, 1969). This may explain why smokers are more prone to lung infections. Cough and phlegm production are observed to diminish or disappear with a reduction or cessation from smoking (Higgins, 1991).

Moreover, other diseases of the circulation such as ischemic disease (Abbott, 1986; Darby, 1989; Doll, 1964; Ernst, 1987; Lowe, Lee, 1992; Zalokar, 1981) and peripheral vascular disease (Ciuffetti, 1989b) have been related to cigarette smoking. Furthermore, smoking during pregnancy has been associated with babies small for their dates, and more frequent still-births and deaths perinatally (Lowe, 1959).

1.7.3 THE COMPOSITION OF CIGARETTE SMOKE

Tobacco smoke has a complex composition (Frei, 1991; Halliwell, 1989; Heseltine, 1987; Royal Coll Phys, 1971). At least 2500 compounds have been identified, some of which are listed in Table 1.1, including constituents of tobacco itself and also chemicals added during harvesting and processing (Heseltine, 1987). Moreover, substances are now being added to the paper used to wrap the cigarettes (Heseltine, 1987).

TABLE 1.1

CONSTITUENTS OF CIGARETTE SMOKE

TOTAL PARTICULATE MATTER

carbon monoxide
nicotine
nitrogen oxides
2-nitropropane
hydrogen cyanide

ALCOHOLS

butanol-1
butanol-2
ethanol
methanol
2-methylpropanol-1
propanol-1

POLYNUCLEAR AROMATIC COMPOUNDS

anthanthrene
anthracene
benzo[a]antracene
benzo[b]fluoranthene
benzo[j]fluoranthene
bemzo[k]fluoranthene

benzo[ghi]fluorene
benzo[b]fluorene
bezo[ghi]perylene
benzo[c]phenanthrene
benzo[e]pyrene
carbazole
chrysene

coronene
dibenz[a,h]acridine
dibebnz[a,j]acridine
dibenz[a,c]anthracene
dibebz[a,j]anthracene
7 H-
dibenzole[c,g]carbazole
dibenzo[a,e]pyrene
dibenzo[a,h]pyrene
dibenzo[a,i]pyrene
dibenzo[a,l]pyrene
dimethylphenanthrene

AMMONIA AND VOLATILE AMINES

ammonia
dimethylamine
2,5-dimethylpyrazine
ethylamine
methylamine
methylpyrazines
2-, 3- ,4-methylpyridines
1-methylpyrrolidine

pyridine
pyrrolidine
trimethylamine
hydrazine

PHENOLIC COMPOUNDS AND QUINONES

catechol

4-ethylcatechol
ethylphenols
eugenol
guaiacol
hydroquinone
3'-hydroxyisoeugenol
isoeugenol

3-, 4-methylcatechol
1-napthol
2-napthol
phenol
resorcinol
4-vinylcaechol
4-vinylguaiacol

2-, 3-, 4-vuinylphenols
xylenols
ortho-, meta-, para-cresol

AROMATIC AMINES

2-, 3-, 4-aminobiphenyls
aniline
2-, 3-, 4-ethylanilines
2-methyl-1-napthylamines
1-, 2-napthylamines

VOLATILE ALDEHYDES AND KETONES

acetaldehyde
acetone
acrolein
crotonaldehyde
formaldehyde
furfural

OTHER VOLATILE COMPOUNDS

benzene
urethane
vinyl chloride

PYRIDINES AND PYRAZINES

2,3-dimethylpyrazine
2,4-, 2,5-, 2,6-lutidines

2-methylpyrazine
3-methylpyridine
2-, 3-, 4-picolines
pyridine
2-vinylpyridine
n-nitrosamines
4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone
N'-nitrosoanabasine
N'-nitrosoanatabine
N-nitrosodi-*n*-butylamine
N-nitrosodiethanolamine
N-nitrosodiethylamine
N-nitrosidimethylamine
N-nitrosomethylethylamine
N'-noricotine
N-nitrosopiperidine
N-nitrosopyrrolidine

METALS

aluminium
antimony
arsenic
bismuth
cadmium

TABLE 1.1 CONTINUED

fluoranthene

fluorene

indeno[1,2,3-sd]pyrene
1-, 2-, 3-, 4-, 5-, 6-metylchrysenes
2-, 3-methylfluoranthenes
1-methylphenanthrene
perylene
phenanthrene
pyrene

triphenylene

CARBOXYLIC ACIDS

acetic acid

benzoic acid
n-butyric acid
formic acid
lactic acid
phenylacetic acid
propionic acid
succinic acid

ortho-, meta-, para-toluidines
2,3-, 2,4-, 2,5-, 2,6-dimethylanilines

LACTONES

coumarin
γ-butyrolactone

AGRICULTURAL CHEMICALS

captan
carbaryl
para, para'-,and ortho, para'-DDD
para, para'-,and ortho, para'-DDT
endrin
malathioin
maleic hydrazide
thiodan

caesium

chromium

cobalt
copper

gold
iron
lanthanum
lead
magnesium

manganese
mercury
nickel
potassium

scandium

selenium
silver
sodium
telluriam
zinc

polnium-210
lead

from (Heseltine, 1987).

The more important components associated with pulmonary disorders are:

- substances that can cause cancer
- irritants that may cause bronchitis
- carbon monoxide which interferes with the blood's capacity to carry oxygen.
- nicotine which may have adverse effects especially on the nervous system and on the heart and blood vessels

Cancer promoting and cancer initiating substances are present in cigarette smoke (Halliwell, 1989; Heseltine, 1987; Royal Coll Phys, 1971). The particulate matter, which when collected on filters is called cigarette smoke condensate, has been associated with cancer in animal studies where such condensates are carcinogenic to animal skin or dog trachea (Davies, 1969; Tobacco Research Council, 1969). Moreover, several years after a reduction in the tar levels of cigarettes by manufacturers, a decrease in lung cancer rates became apparent (Heseltine, 1987; Williams, 1991).

Irritants in cigarette smoke induce coughing and narrowing of the bronchi (Anderson, 1962), a ciliastatic effect (Tobacco Research Council, 1969) and also stimulation of the bronchial glands to increase mucus production (Anderson, 1962) thereby interfering with the lung's cleansing mechanism.

The high concentrations of carbon monoxide in cigarette smoke, which has a greater affinity for haemoglobin than oxygen, reduces the smoker's capacity to transport oxygen (Goldsmith, 1968). As cigarette smokers inhale more than cigar or pipe smokers, their blood carbon monoxide levels are higher. To compensate more blood must be pumped around the body, placing a strain on the heart. The lack of oxygen may also interfere with the growth of an unborn child and diminish athletic performance of smokers (Lowe, 1959).

As nicotine is probably the cause of the smoking habit, it's content in cigarette smoke and absorption is important. Nicotine in the alkaline smoke of cigars and pipe tobaccos is absorbed through the lining of the mouth and pharynx, but nicotine in the acid smoke of cigarettes is absorbed from the lungs (Levy, 1989). Thus cigarette smokers inhale more than pipe or tobacco smokers (Kershbaum, 1967), which explains why disease is more prevalent in cigarette smokers than cigar or pipe smokers (Levy, 1989). Nicotine can have a stimulating or sedating effect depending on the dose and the activity of the smoker (Royal Coll Phys, 1971). The alkaloid affects the nervous system by causing the release of noradrenaline and adrenalin

from the adrenal glands (Armitage, 1965), and thereby causing an increase in heart rate, and hence blood pressure, and constriction of the blood vessels (Klein, 1983).

THE OXIDISING POTENTIAL OF CIGARETTE SMOKE

The free radical content of cigarette smoke is also proposed to have a causative role in pulmonary diseases, particularly emphysema (Blue, 1978). Cigarette smoke is known to contain many reactive oxidants (acetaldehyde, acrolein, cyanide, isoprene, toluene, acetonitrile, peroxides, epoxides, peroxyl radicals, carbon monoxide, nitrous oxide, nitrogen dioxide, ozone etc)(Church, 1985; Dalhamn, 1968; Eichel, 1969). The free radical species contained in cigarette smoke have been extensively studied by Pryor, Church and their coworkers (Church, 1985; Pryor, 1983; Pryor, 1976b). Pryor's group have established that the tar and the vapour phase in a puff of cigarette smoke each contains about 10^{14} free radicals (Pryor, 1983; Pryor, 1976b). The radicals present in the particulate fraction of cigarette smoke are much less reactive than vapour phase radicals, but persist for longer with a half-life of 10 hours (Pryor, 1983; Pryor, 1976b). The vapour phase of cigarette smoke contains mainly oxygen-centered free radicals that have a shorter half-life of 5 minutes in the vapour phase and 1 minute in aqueous solution (Pryor, 1983; Pryor, 1976b). However, the radical concentration of vapour phase smoke was not significantly decreased when drawn 180 cm down a glass tube (Pryor, 1983; Pryor, 1976b). This is particularly relevant when considering what happens in the lungs.

Moreover, the radicals in smoke can react either with oxygen or each other to form further radicals (Borland, 1985; Halliwell, 1989; Tsuchiya, 1992). For example nitric oxide can react with oxygen to form the radical nitrogen dioxide. Both nitric oxide and nitrogen dioxide may react with hydrogen peroxide to form hydroxyl radicals (Borland, 1985). In this manner radicals present in cigarette smoke can also interact with radicals produced by phagocytes in the lung. However, free radicals can also combine to give non-radical products thus establishing a steady-state concentration of radicals.

Biotransformation of smoke components in the respiratory tract can also occur (Cohen, 1990). Cells in the respiratory tract, such as the Bowman's gland cells, bronchiolar non-ciliated (CLARA) cell and Type II epithelial cells, can transform inhaled chemicals into active metabolites which may then be detoxified and excreted. However, the interaction may result in products which damage the cells themselves (Richards, 1992). Richards (1992) was able to demonstrate that although glutathione can protect CLARA cells from damage, by first bubbling the smoke

through a solution of GSH, the smoke was more damaging to the cells suggesting that one or more component of the cigarette smoke had reacted with GSH to produce a reactive metabolite.

Some investigators propose that the acetaldehyde (Goekas, 1984), acrolein and cyanide (Dalhamn, 1966) content of smoke are the most potent substances in the vapour phase, and have therefore tested the toxicity of these compounds in isolation on cell viability and function. However, as described above, the interaction of smoke components may play an important part in its toxicity. Thus experiments in which animals or cells are exposed to isolated smoke components may yield results not applicable to whole smoke (Borland, 1985). Although this would also apply to studies using only the vapour phase of cigarette smoke, some cells such as the blood cells and the pulmonary endothelium may be exposed, *in vivo*, only to the vapour phase components (Borland, 1985).

1.8 THE MAJOR FACTORS AFFECTING NEUTROPHIL SEQUESTRATION IN THE LUNGS

At least three mechanism for leucocyte sequestration have been proposed: haemodynamic forces; adherence of leucocyte to the endothelium; or sequestration due to the biophysical properties of the cell (size and ability of the cell to deform) and the capillary bed.

1.8.1 HAEMODYNAMICS

As the shear stress of blood is the only force available to drive leucocytes through the narrow capillaries, a reduction in blood flow would increase cell transit time. This has been demonstrated *in vitro* (Downey, 1988) and observed *in vivo*, particularly in ischemic tissue (Dahlgren, 1984; Engler, 1983; Nash, 1988c; Schmid-Schonbein, 1990). Early studies by Atherton and Born (Atherton, 1973) demonstrated a correlation between the rolling of leucocytes in hamster cheek pouch or mouse mesentery and blood velocity, more so than adhesion to vascular endothelium. In the pulmonary circulation in dogs, a reduction in local blood flow, following the administration of complement factors, was found to contribute to neutrophil retention (Lien, 1991; Lien, 1990). By increasing or decreasing the pulmonary blood flow, neutrophil transit times were shifted to shorter or longer times respectively (Lien, 1990). Likewise, Braide and colleagues (1991) found that in isolated but ventilated rat lung preparations the retention of leucocytes depended on

the flow rate of the perfusate. Similarly, various studies from Hogg's group in Vancouver demonstrate that haemodynamic forces can influence leucocyte pulmonary transit. Martin and associates (1982) found an enhanced neutrophil retention in dog lungs with decreasing blood flow. They subsequently demonstrated that a difference in blood flow was evident in the upper compared with the lower regions of the lungs which contributed to the extent of neutrophil sequestration in each region (Martin, 1987). Doerschuk and associates (1987) reported similar kinetics for neutrophil transit relative to erythrocytes in rabbit lungs, and analogous observations for the retention of monocytes, lymphocytes, and platelets (Doerschuk, 1990a). Moreover, haemodynamic forces were found to influence the transit of isolated neutrophils through capillary sized pores *in vitro* (Downey, 1988).

To maintain blood flow across the lungs, an increase in resistance in some of the capillaries, caused by the slower transitting leucocytes, could be compensated for by an increased blood flow and thus erythrocyte transit in the remaining capillary segments (Hogg, 1988; Schmid-Schonbein, 1980c; Warnke, 1990; Warnke, 1992). As acute cigarette smoking increased erythrocyte transit, yet also increased neutrophil sequestration (MacNee, 1989d), a change in haemodynamics is unlikely to be the mechanism of the enhanced neutrophil sequestration which results with acute smoking.

1.8.2 LEUCOCYTE ADHESION

In humans, interactions between leucocytes and endothelial cells are mediated by at least three adhesion receptors: the integrins, the immunoglobulin-related and the selectin molecules.

LEUCOCYTE INTEGRINS

The leucocyte integrins were the first of the adhesion receptors identified to mediate leucocyte-endothelial cell adhesion (Beatty, 1983; Sanchez-Madrid, 1983). This family of leucocyte adhesion molecules (leu-CAM) consists of three surface membrane heterodimeric glycoproteins with distinct α-subunits, named LFA-1, Mo1 or MAC-1 and p150.95, non-covalently associated with a common β_2-subunit. The individual α-subunits have been designated CD11a, CD11b, and CD11c respectively, and the common β_2-subunit CD18 by the Third International Workshop on Leucocyte Differentiation Antigens (Shaw, 1987).

The CD11/CD18 glycoproteins are expressed only on leucocytes. CD11a/CD18 is constitutively expressed, with little or none stored intracellularly, on all normal, non-activated leucocytes but mainly by T and B lymphocytes (Arnaout, 1985). Granulocytes, monocytes, macrophages and natural killer cells express both CD11b/CD18 and CD11c/CD18 (Arnaout, 1990). Circulating granulocytes and monocytes store significant amounts of CD11b/CD18 and CD11c/CD18 in the secondary and tertiary granules in granulocytes, and in intracellular vesicles and peroxidase-negative granules in monocytes (Miller, 1987). Tissue macrophages do not contain intracellular stores of any of these glycoproteins (Miller 1987).

Within minutes of activation, increased cell-surface expression of CD11b/CD18 and CD11c/CD18 occurs as the intracellular granules translocate to, and fuse with, the plasma membrane of the cell (Miller, 1987; Hughes, 1992). However, increased expression of these receptors upon activation is neither necessary nor sufficient for cell adhesion to endothelial cells (Vedder, 1988a). Phosphorylation of the CD18 subunit appears to be necessary before these adhesive glycoproteins are capable of adhesive interactions (Chatila, 1989).

The importance of the β_2-integrins in host defense is evident in patients with the rare inherited disease, leucocyte adhesion deficiency (LAD)(Anderson, 1987; Davies, 1991). These patients do not express CD18 on their leucocytes. Therefore, due to impaired leucocyte adhesion, they exhibit recurrent infections (Anderson, 1987). One case reported for a man with LAD is of particular interest. The patient, as is typical for LAD, was found to have large numbers of intravascular neutrophils at postmortem examination, but few neutrophils in the lung interstitium or alveoli despite a pulmonary infection (Davies, 1991). A white cell scan revealed a prolonged intravascular survival time of 22 hours for indium-labelled neutrophils in this patient (Davies, 1991) compared with 6-9 hours for normal subjects (Athens, 1961a; Cartwright, 1964) The reinjected neutrophils, however, localised normally in the lung, liver and spleen following reinjection, and were observed to demarginate when the patient was exercised at 50 minutes (Davies, 1991) which suggests CD18-mediated adhesion is not involved in normal neutrophil margination.

IMMUNOGLOBULIN SUPERGENE FAMILY

The immunoglobulin supergene family function in cell-cell recognition or adhesion. This family, of which there are at least 10 members, share a common immunoglobulin domain of 90 and 100 amino acids in a sandwich of two sheets usually linked by a disulfide bond. Three members of this family are presented on epithelial and

endothelial cells and play a role in leucocyte adhesion: intracellular adhesion molecule-1 (ICAM-1, CD54), ICAM-2, and vascular cell adhesion molecule (VCAM-1). Also, leucocytes express the ligand ICAM-3 (CD50) (Fawcett, 1992).
ICAM-1 is present at low levels on endothelial and epithelial cells. It can be markedly upregulated by inflammatory stimuli such as interleukin-1, endotoxin, leukotriene B_4 (Vedder, 1988a) and tumour necrosis factor. Expression of ICAM-1 peaks after 24 hours, suggesting mRNA and protein synthesis is required (Dustin, 1986). ICAM-2 is basally expressed on endothelial cells and can not be induced (Fougerolles, 1991). It's role is still unknown. VCAM-1 is an inducible glycoprotein which mediates lymphocyte adhesion with a similar time response as ICAM-1 (Osborn, 1989). ICAM-3 is constitutively expressed at high levels on resting leucocytes of all lineage, but with no expression on either resting or cytokine activated endothelial cells. It is closely related to ICAM-1, consisting of 5 immunoglobulin domains, and likewise binds CD11a (Fawcett, 1992).

The involvement of the leucocyte integrins for binding to endothelial cells has been demonstrated both *in vivo* and *in vitro* using monoclonal antibodies directed at specific epitopes on CD11/CD18 subunits (Arfors, 1987; Horgan, 1990; Lindbom,, 1990; Lucinska, 1989; Vedder, 1988a & 1988b; Yoder, 1990). Leucocytes adhere to unstimulated endothelial cells under static conditions (Hughes, 1992; Lawrence, 1990; Lindbom, 1990; Lucinska, 1989). Activation of leucocytes with chemotactic factors such as fMLP, $C5_a$, and LTB_4 significantly increased this cell-cell adhesion (Hughes, 1992; Lawrence, 1990; Lindbom, 1990), which was inhibitable by monoclonal antibodies against CD18 or ICAM-1 (Lawrence, 1990; Lindbom, 1990; Lucinska, 1989).

THE SELECTINS

As well as the firm adhesion of leucocytes to endothelial cells, the rolling of leucocytes along the endothelium has also been observed (Atherton, 1973; Schmid-Schonbein, 1980b). Leucocyte rolling does not appear to involve leucocyte integrins but specific cell-surface receptors interactions. To date three such receptors, named LEC-CAMS (lectin like adhesion molecules) or selectins, have been identified: L-selectin (lymphocyte homing receptor LECAM-1, LAM-1, MEL-14) found on leucocytes, and E-selectin (ELAM-1) and P-selectin (GMP-140, PADGEM, CD62) present on endothelial cells. They share a common molecular structure consisting of a lectin-like domain in the N-terminal region of the protein, an epidermal growth factor domain, a series of complement-regulatory domains, a transmembrane domain, and

a short cytoplasmic tail (Albeda, 1991). The name selectins represents the selective cell-cell interactions of these receptors, and also that they mediate lectin-type interactions on target cells.

L-selectin is inherent on the surface of approximately 70% of circulating lymphocytes and also on neutrophils and monocytes (Tedder, 1990). However, in response to activation, L-selectin is rapidly shed (von Adrian, 1992). L-selectin on neutrophils, but not lymphocytes, interacts with both P-selectin and E-selectin (Bevilacqua, 1989; Lawrence, 1991; Picker, 1991).

E-selectin and P-selectin are found on the surface of endothelial cells. E-selectin is not present in unstimulated endothelium but synthesised *de novo* upon cell stimulation by inflammatory cytokines such as interleukin-1, tumour necrosis factor, or endotoxin with peak expression around 4-6 hours, declining by 12-24 hours (Bevilacqua, 1989). E-selectin binds to circulating cells via a carbohydrate ligand called the sialyl-Lewis X which is expressed on neutrophils and monocytes, but not lymphocytes or erythrocytes (Picker, 1991).

P-selectin is expressed on endothelial cells and also on platelets. Unlike E-selectin it is intrinsically expressed on normal tissue. It is also stored in α-granules in platelets and Weibel-Palade bodies of endothelial cells and can be rapidly induced to the cell surface within minutes of activation with histamine, thrombin or platelet activating factor (McEver, 1989). The ligand for P-selectin is also the carbohydrate sialylated Lewis X found on the surface glycoproteins and glycolipids of leucocytes (Polley, 1991).

LEUCOCYTE STATIC ADHESION UNDER CONDITIONS OF FLOW

Under conditions of flow, adherence of fMLP stimulated neutrophils to untreated endothelial cells was only observed at low wall shear stresses (Lawrence, 1990; Perry, 1992; Worthen, Smedley et al., 1987), below those estimated to occur *in vivo* in postcapillary venules (from 5 to 50 dyn.cm^{-2})(Atherton, 1972; House, 1989; Schmid-Schonbein, 1980b; von Adrian, 1992) except for low flow regions of the vasculature and in near stasis conditions as may occur locally in acute inflammation. However, IL-1 stimulation of endothelial monolayers *in vitro* did enhance the adherence of fMLP-treated leucocytes under flow conditions (at 0.5 dynes.cm^{-2})(Lawrence, 1990).

Although *in vivo* observations also suggest that adherence is dependent on shear stress (Atherton, 1973; von Adrian 1992; Worthen, Smedley, 1987), Perry and Granger (1991) noted a higher shear stress was required to abolish adherence of leucocytes in cat mesentery *in vivo* (~8 dyne.cm^{-2}) than *in vitro* (1-2 dyne.cm^{-2}). In

fact, they observed adhesion *in vivo* at shear rates which are normal in cat mesentery. These authors suggested the lower shear stresses required to prevent adhesion *in vitro* may be due to the use of isolated leucocytes, where the isolation procedure may have interfered with the CD11/CD18 glycoproteins; the use of cultured endothelial cells where likewise the ligand for CD11/CD18 may be altered; or possibly that the medium used *in vitro* was unlike the *in vivo* conditions of a complex mixed cell suspension (Perry and Granger, 1991). Despite the effects of shear stress, Perry and Granger (1991) and also Arfors and colleagues (1987) were able to demonstrate that leucocyte adhesion to endothelium *in vivo* was CD11/CD18 mediated. Likewise, in a rabbit model, *intra vital* studies by Lindbom and associates (1990) showed inhibition of neutrophil adhesion in muscle tissue exposed to zymosan activated serum in anti-CD18 treated animals.

High shear stresses in arterioles compared with capillaries and venules has been suggested as the reason for the lack of adhesion in arterioles. However, Perry and Granger (1991) demonstrated there was still a marked difference in leucocyte adhesion between the arterioles and venules at the same shear rate, and thus proposed a heterogenous distribution of adhesion receptors between the vessel types.

LEUCOCYTE ROLLING ADHESION UNDER CONDITIONS OF FLOW

Intra vital studies have also observed leucocytes rolling along the endothelium of small venules. Such rolling may be initiated when leucocytes enter the postcapillary venules and/or by the interaction with erythrocytes as described above. Whether this is a normal physiological phenomenon, or reflects activation of leucocytes or endothelium due to the experimental procedure is a matter of debate. Nevertheless, this rolling appears to be the first interaction of leucocytes and the vascular endothelium (Lawrence, 1990; von Adrian, 1992).

Lawrence and associates (1990) found that, whereas adhesion to unstimulated endothelial cells at low flow was inhibited by anti-CD18, the interaction of fMLP treated leucocytes with IL-1 stimulated endothelium was not altered by anti-CD18. This suggested a CD18-independent mechanism was also involved. In contrast, transendothelial migration of neutrophils was inhibited under these conditions and therefore appears to be CD18 mediated (Lawrence, 1990).

In vitro studies, by Lawrence and Springer (1991) using artificial bilayers containing both P-selectin and ICAM-1, demonstrated that interaction with ICAM-1 was not possible unless there was a rolling interaction with P-selectin. Their data confirmed earlier *in vitro* studies by Ley and associates which indicated that leucocyte rolling is

a necessary precondition for leucocyte fast adhesion to cultured endothelium under shear conditions (Ley, 1989). The rolling action on P-selectin did not cause activation of the leucocyte CD11/CD18 integrins (Lawrence, 1991). Rolling of neutrophils under normal shear flow can be inhibited by preincubation with anti-L-selectin antibodies; the shedding of L-selectin upon cell activation; or treatment with chymotrypsin to remove the L-selectin from the neutrophil surface, but not by anti-CD18. Lindbom (1992) and von Adrian (1992) and their co-workers found that chemotactic stimulation shed L-selectin, reduced leucocyte rolling and caused a proportional decrease in leucocyte firm adhesion.

That a slowing of leucocytes via selectins was necessary for firm adhesion under the shear flow conditions *in vivo* has also been observed. Von Adrian and colleagues (1991) observed the rolling and adhesive behaviour of fluorescently labelled human neutrophils injected into rabbit mesentery. Although the removal or inhibition of L-selectin did not alter CD18 expression or adhesion under static conditions, under flow neutrophils were unable to adhere to venular endothelial cells (von Adrian, 1991). Even by enhancing the adhesive interaction by endothelial activation with IL-1, CD18 was unable to initiate leucocyte-endothelial adhesion under conditions of flow (von Adrian, 1992). Moreover, by inhibiting the L-selectin interaction with, and thus leucocyte rolling along the endothelium, by infusion of sialyl Lewis X (a ligand for P-selectin), cobra venom-induced lung injury in the rat was markedly reduced (Mulligan, 1993). These studies, therefore, propose the concept that leucocyte-endothelium interaction *in vivo* is mediated sequentially by two leucocyte adhesion molecules.

LEUCOCYTE ADHESION IN THE PULMONARY CIRCULATION

The *in vivo* studies detailed above are observations made in the systemic circulation. Leucocyte-endothelial interaction may be different in pulmonary vasculature. The *in vitro* investigations and observations made in the systemic circulation have established that the adhesion of neutrophils is highly dependent on shear stress. Blood pressure in the pulmonary microvasculature is lower than the systemic circulation (von Euler, 1947), thus CD18-mediated adhesion may be more likely to mediate the sequestration of neutrophils in normal lungs. Yoder and colleagues (1990) observed, by *intra vital* microscopy of the dog lung, that the normal, unstimulated transit of neutrophils was CD11/CD18-independent. However, the authors noted a monoclonal antibody (Mab) to the CD11/CD18 adhesion glycoprotein prevented neutrophil sequestration following activation by prior infusion of zymosan activated plasma (ZAP) through a pulmonary arterial catheter,

which suggests CD18-mediated adhesion only plays a role in inflamed lungs. Likewise, Horgan and associates (1990) found ischemia-reperfusion injury in the lungs of rabbits could be prevented by treatment with anti-CD18 Mab prior to reperfusion. In contrast, Vedder and associates (Vedder, 1988b) showed CD18 Mab was ineffective at reducing lung injury following, in essence, whole body ischemia-reperfusion in rabbits, whilst effectively preventing injury to the liver and gastric mucosa in the same animal. Moreover, Lundberg and Wright (1991) found that a transient fMLP-induced neutrophil lung sequestration was not inhibited by pretreatment with an antiCD18 antibody. These contradictory data may be explained by the finding of Doerschuk and coworkers (Doerschuk, 1990b).
To determine whether adhesion in the pulmonary circulation differed from that of the systemic circulation, Doerschuk and colleagues (1990b) performed a study comparing neutrophil-endothelial adherence in the pulmonary and systemic circulation for various stimuli. Neutrophil emigration was found to be CD18-dependent in the systemic circulation (abdominal wall and peritoneal cavity) for all stimuli tested. In contrast, neutrophil adhesion to endothelial cells and emigration in the lungs was found to be either CD18-dependent or independent, specific to the stimuli. Installation of *S pneumoniae* or hydrochloric acid into rabbit lungs induced CD18-independent neutrophil emigration, as anti-CD18 pretreatment of the animals did not block cell migration. Endotoxin or PMA instillation was shown to be CD18-dependent. Thus CD18 mediated adhesion appears to be both site and stimulus dependent.

However, Walsh and coworkers (1991) found leucocyte-induced acute lung injury associated with gram-negative sepsis was both CD18-independent and CD18-dependent. Likewise, Doerschuk (1992) demonstrated a CD18-independent and -dependent phase for neutrophil adhesion to one stimulus. During continuous infusion of ZAP in the dog neutrophil accumulation was initially CD18-independent, lasting between 5 and 7 minutes, with a CD18-dependent mechanism accounting for the prolonged sequestration after 15 minutes of ZAP infusion. The rapid CD18-independent neutrophil lung sequestration may be due to an alternative adhesion system such as the selectins, or due to a decreased cell deformability. Indeed the study by Mulligan and associates (1992) which demonstrated that the cobra-venom-induced acute vascular injury in the rat lung was diminished by simultaneous infusion of an anti-P-selectin antibody, suggests a role for the selectins for leucocyte adhesion in the lungs.

LEUCOCYTE ADHESION AND CIGARETTE SMOKE EXPOSURE

In general, little is known about the effect of cigarette smoke on leucocyte adhesivity. Klut and associates (1993) found increased expression of CD18 and a reduced expression of L-selectin on intravascular neutrophils in the lungs of rabbits following acute smoke exposure, which suggests increased leucocyte adhesivity. In contrast, Selby and colleagues (1992) found both the spontaneous and stimulated adhesion of neutrophils was reduced by *in vitro* smoke exposure. Also, alveolar macrophages obtained from smokers were found to be less adherent to nylon fibers than those from non-smokers (Rasp, 1978). Moreover, the increased blood flow measured during acute cigarette smoking (MacNee, 1989d) is likely to hinder neutrophil-endothelial adhesive interaction as leucocyte adhesion *in vivo* is inversely related to shear stress (Atherton, 1973). Thus increased neutrophil adhesivity does not appear a likely mechanism for the increased pulmonary sequestration observed during smoking.

1.8.3 LEUCOCYTE PULMONARY SEQUESTRATION AND DEFORMABILITY

For each circulation of the body, all circulating leucocytes must pass through the pulmonary microvasculature. As detailed above, the size discrepancy between the neutrophil and the pulmonary capillaries necessitates deformation of the cell. As such it is well established that cell deformability is a major influence on neutrophil transit of the microvascular bed under normal conditions (Bagge, 1977; Chien, 1985; Schmid-Schonbein, 1980b).

Enhancement of this normal leucocyte pulmonary sequestration has been observed following exposure (by installation or inhalation into the lungs, or intravenous administration) to a range of inflammatory mediators such as fMLP, C_{5a}, endotoxin, granulocyte-macrophage colony-stimulating factor (GM-CSF) platelet activating factor (PAF), LTB_4, and tumour necrosis factor (TNF) both in animals and man (Devereux, 1987; Haslett, 1987; Lien, 1987 & 1991; Martin, 1989; Peters, 1992). These mediators are known to activate leucocytes (Berkow, 1987; Khwaja, 1992; Packman, 1990; Shalit, 1987; van der Poll, 1992; van Zee, 1991), causing stiffening of the cells (Nash, 1988a; Worthen 1989) and shape change (Howard, 1990; Roos, 1987; Wallace, 1984).

An impaired flow of leucocytes has also been reported in clinical conditions associated with cell activation such as tissue ischemia (Ciuffetti, 1989a & 1989b; Engler, 1983; Laurient-Roudaut, 1981; Nash, 1988c; Neumann, 1990). In low flow

states, such as in ischemic tissue and possibly in the lung capillaries, a vicious cycle can be envisioned of leucocytes obstructing vessels, being activated to release free oxygen radicals and proteases, and thereby causing endothelial damage which in turn leads to trapping of leucocytes and further damage.

CIGARETTE SMOKE AND CELL DEFORMABILITY

Cigarette smoking causes an inflammatory reaction with increased numbers of leucocytes present in the circulation and airways (Bridges, 1977; Corberand, 1979; Gadek, 1979; Hunninghake, 1983; Ludwig, 1982; Noble, 1975; Richards, 1989). Moreover, these leucocytes appear to be activated or in a primed condition (Corberand, 1979; Gillespie, 1987; Hoidal, 1982; Ludwig, 1982; Richards, 1989). Hence, the enhanced sequestration observed during smoking (Bosken, 1991; MacNee, 1989d) could be due to a reduction in neutrophil deformability caused by cell activation by cigarette smoke exposure. Although cigarette smoking has been associated with clinical conditions in which leucocyte deformability is reduced (Ciuffetti, 1989b; Ernst, 1987; Lowe, 1992), a direct effect of smoke exposure on leucocyte deformability has not been demonstrated.

The only report to date for an effect of cigarette smoke exposure on cell deformability found a reduced deformability for human and hamster alveolar macrophages following exposure to whole, but not to vapour phase cigarette smoke *in vivo* (Smith, 1986). This was suggested to result from an increase in cell size and loss of membrane surface area following phagocytosis of particulate matter.

1.9 AIMS

The overall aim of this thesis was to determine the mechanism of the enhanced pulmonary sequestration of neutrophils observed *in vivo* during acute cigarette smoking in man (MacNee, 1989d). Such leucocyte sequestration in the pulmonary vasculature could be influenced by three factors: the dynamics of blood flow, leucocyte and endothelial adhesivity, and the mechanical properties of leucocytes. The preceding review of the literature has considered each of these areas with regard to the pulmonary circulation and the known effects of cigarette smoking. From the studies discussed, a reduction in neutrophil deformability would appear the most probable mechanism for the smoke-induced sequestration.

Haemodynamic factors were chiefly dismissed as erythrocyte lung transit time, reflecting blood velocity, was found to be increased during smoking (MacNee, 1989d). Neutrophil adhesivity, although a major influence, does not appear to be the

initiating factor for neutrophil sequestration in the lungs, particularly under conditions of flow. Hence the main focus of this thesis was to investigate the effect of cigarette smoke exposure on the neutrophils' ability to deform.

In chapter 2 the techniques general to all the work in this thesis are established. These include the method for acquiring quiescent, pure neutrophil populations, establishing a system for measuring cell deformability, and also for the *in vitro* smoke exposure of cells.

In chapter 3 the *in vitro* measurement of cell deformability was compared with the first pass transit (initial sequestration) of neutrophils through the pulmonary microcirculation in man.

The effect of *in vitro* vapour phase cigarette smoke exposure on the deformability and function of harvested neutrophils was investigated in chapter 4.

Chapter 5 describes studies of the mechanism of changes in neutrophil deformability and function.

In chapter 6 the *in vivo* effect of cigarette smoking on leucocyte deformability was assessed by measuring the filterability of whole blood *in vitro*. Blood samples were also tested for evidence of an oxidant stress caused by acute smoking.

A summary of the findings, and the overall conclusions which can be drawn from the data in this thesis, can be found in chapter 7, with suggestions for future studies.

CHAPTER 2
METHODS GENERAL TO THE WORK IN THIS THESIS

2.1 INTRODUCTION

Blood contains several types of cells which perform very different functions, ranging from the transport of oxygen and carbon dioxide by erythrocytes, to the removal and digestion of debris and combating infection by leucocytes. In addition platelets function to aid blood clotting, and hence blood flow at sites of injury. As well as a functional difference, the cellular constituents of blood have different physical characteristics which independently, and by their interaction, influence the flow of blood, i.e. blood rheology particularly in microcirculatory beds as detailed in chapter 1.

Evidence for a pathogenic role for neutrophils in tissue damage (Henson, 1987; Malech, 1987; Weiss, 1989) has encouraged *in vitro* studies of the physical characteristics and function of these cells. Although such studies can be performed on unfractionated blood samples, the characterisation of the different cell types is best performed on isolated subpopulations of cells. Moreover, interest in the distribution of neutrophils in the vascular system, in normal and inflammatory conditions, has lead to the radiolabelling of cells for non-invasive external imaging (Haslett, 1987; Hogg, 1987; MacNee, 1990 & 1993; Muir, 1984; Peters, 1985a & 1985b).

Hence, in this chapter neutrophil function was assessed to demonstrate the functional integrity and quiescence of the cells isolated from whole blood. Also, as the aim of this thesis was to investigate the effect of cigarette smoke exposure on neutrophil deformability, the systems for *in vitro* smoke exposure and the measurement of cell deformability (a constant flow filtration system) were assessed for reproducible delivery of a dose of smoke and filtration measurement respectively.

2.1.1 NEUTROPHIL ISOLATION FROM WHOLE BLOOD

THE HISTORY OF CELL HARVESTING

Much progress has been made since Rabinowtz in 1964 isolated neutrophils from whole blood and other leucocytes by layering leucocyte rich plasma (obtained by dextran sedimentation) onto columns of glass beads. The neutrophils adhered to the glass beads and were washed off with EDTA (Rabinowtz, 1964). Today various cell harvesting techniques are available depending on the cell type of interest, purity required and time available.

A mixed leucocyte population can be obtained rapidly from whole blood, with minimal cell handling, by sedimentation of the erythrocytes. By mixing blood with a compound which aggregates erythrocytes, their sedimentation rate is increased, whereas the sedimentation of leucocytes is only slightly affected. The leucocytes can then be collected from the upper part of the tube when the erythrocytes have settled to the base. In the early sixties Boyum (1964) adapted this principle and introduced density gradients to isolate leucocytes from erythrocytes. Without actually mixing blood with an aggregating agent, blood was carefully layered on top of a mixture of an agglutinating compound and a compound of high density, such as sodium metrizoate. Erythrocyte aggregates were then formed at the interface, and sedimented to the bottom of the tube leaving the leucocytes in the plasma layer. Several years later Boyum (1968) introduced a means of obtaining pure leucocyte sub-populations from whole blood. He utilised the principle (Stokes law) that the rate of sedimentation is zero when the cell encounters a medium of identical density and thus by gravity, or the application of a centrifugal force, cells can be separated by their density. Boyum thus developed a one-step technique by which lymphocytes were separated from whole blood using a Ficoll-Hypaque density gradient (density 1.077 $g.ml^{-1}$) and centrifugation. This technique was subsequently modified to separate the granulocytes from erythrocytes (English, 1974; Ferrante, 1978).
The introduction of Percoll, a colloidal media made up of silica particles covered in polyvinylpyrrolidine (manufacturers information), has an advantage over Ficoll-Hypaque in that the density (1.13 $g.ml^{-1}$) can be adjusted to produce a range of densities by the addition of 0.25 M sucrose, balanced salt solutions or plasma. This allows the production of multiple layers of discontinuous densities to match the densities of the different cell sub-populations. Moreover, Percoll is non-toxic, iso-osmotic and has a low viscosity which allows it's use with low centrifugation speeds thus minimising the adverse effect of centrifugal force on the cells.

An alternative cell isolation technique, centrifugal elutriation was introduced by Pretlow (1979) and developed in the early seventies by Beckman (Hogg, 1987). Centrifugal elutriation (CCE) works on the principle of flow pressure counteracting centrifugal force such that outward centrifugal pressure is opposed by inward centripetal flow. As the more dense cells and larger cells offer more resistance to the flow acting on them, under controlled conditions platelets, erythrocytes, lymphocytes, monocytes and neutrophils are in turn forced out of the elutriator.

More recently the availability of laser based flow cytometry has provided a means of separating blood cells with extreme purity. In the flow cytometer, as each individual cell breaks the path of a laser beam, the light scatter detected at 90° and 180° can be used to determine the cell type. The light measured at 180° is dependent on the size of the cell, whereas the light deflected at right angles relates to the granular content of the cell. Thus each cell can be accurately characterised by its size and granularity.

The handling involved for any of these isolation procedure could, however, potentially be harmful to these fragile cells. Several studies have, therefore, compared the functional integrity of leucocytes isolated by different techniques (Berkow, 1983; Glasser, 1990; Haslett, 1985; Nash, 1988a).

COMPARISON OF NEUTROPHIL HARVESTING TECHNIQUES

As mentioned, various cell separation methods have been developed over the last three decades, comprising:

- erythrocyte sedimentation
- density gradients
- centrifugal elutriation
- flow cytometry
- hypotonic lysis of erythrocytes.

which can be used either in isolation or in combination.

Erythrocyte agglutinating agents, such as dextran, are used initially to obtain leucocyte rich plasma by sedimenting the erythrocytes. The simple isolation of mixed leucocytes from whole blood by sedimentation of the erythrocytes has the advantage of little cell manipulation and is therefore least likely to perturb the cells. However, dextran was found to cause an increase in the endogenous radical production, measured by nitro blue tetrazolium (NBT) reduction, possibly due to phagocytosis or stimulation of the membrane (Jarstrand, 1979). In contrast, Haslett and co-workers (1985) found leucocytes obtained following dextran sedimentation were functionally unaltered. Similarly, Evans and Kukan (1984) found neutrophil deformability was unaffected by separation in dextran, and Glasser and Fiederlein (1990) reported no change in neutrophil radical production as a result of erythrocyte sedimentation by hydroxyethyl starch, another macromolecule.

However, for most leucocyte studies pure populations are required. To separate the blood into pure white cell subpopulations, either the use of density gradients, counterflow centrifugal elutriation (CCE), or flow cytometry can be used.

The use of density gradients to isolate pure blood cell subpopulations can take time and involves a large amount of cell handling, including repeated pelleting of the cells by centrifugal force. Flow cytometry and CCE allow separation of very pure leucocyte subpopulations (Franzi, 1983; Yasaka, 1981). CCE separates leucocyte subpopulations from whole blood or following density gradient separation with a high yield, purity, and with excellent viability and functional status (Stevenson, 1984). These isolation techniques has the advantage of preparing the cell subpopulations in suspension, thereby reducing the likelihood of altering the native function of these cells from when circulating in the blood. In addition both techniques allow the cells to remain in a physiologic buffer throughout the procedure.

A comprehensive study by Glasser and Fiederlein (1990) compared various harvesting procedures (erythrocyte sedimentation, density gradient separation, CCE and hypotonic lysis), and combinations thereof, with respect to a range of neutrophil functions including random and directional locomotion, chemiluminescence and NBT reduction, phagocytosis and bacterial killing and morphological integrity. These authors could not, from their data, preferentially select any one isolation procedure over the others. Even brief exposure to hypotonic saline, to lyse contaminating erythrocytes, did not adversely affect the range of functions examined (Glasser, 1990). Only cell locomotion demonstrated any discrimination between the harvesting techniques. However, these results were influenced by the presence of erythrocytes, which depressed both random and directional leucocyte locomotion (Glasser, 1990). Ficoll-Hypaque separated neutrophils, with the addition of a hypotonic lysis step, were more chemotactic towards serum than CCE separated cells (Glasser, 1990), although Roth and Kaeberle (1981) found for bovine neutrophils that random migration was lower following Ficoll-Hypaque separation. Berkow and co-workers (1983) also compared the function of neutrophils harvested by CCE and Ficoll-Hypaque. They found CCE separated neutrophils were larger in volume and released more O_2^- and specific granule constituents, lysosyme and vitamin B_{12}-binding protein, upon stimulation than Ficoll-Hypaque separated cells. They suggested the difference in cell volume may be due to the selection of smaller or larger leucocyte subpopulations by either harvesting techniques. However, the authors found negligible numbers of leucocytes in the other cell fractions obtained from the elutriator. Nor was a subset of large leucocytes found in the discarded fraction of dextran sedimented cells. However, simply the selection of a sub-population of

either more or less active neutrophils, by either of the separating procedures, could explain the increased protease and reactive oxidant release. Conversely, the authors proposed a suppressed activation of Ficoll-Hypaque isolated neutrophils due to their exposure to hypotonic conditions which may alter the ionic fluxes of Na^+, Ca^{2+} and K^+ necessary for the membrane associated activation events (Naccache, 1977).

Neutrophils isolated by CCE are manipulated less and, therefore, considered to be the least functionally perturbed of all cells subjected to a harvesting procedure (Berkow, 1983). However, the studies discussed above do not indicate that the CCE is superior to Ficoll-Hypaque density gradients as a means of isolating pure neutrophil populations. However, a comparison of Ficoll-Hypaque and plasma/Percoll density gradients by Haslett and associates (1985) revealed altered neutrophil function following isolation by Ficoll-Hypaque density gradients, which was not observed for plasma/Percoll harvested neutrophils. They reported priming of Ficoll-Hypaque prepared neutrophils, evident by reduced chemotaxis towards fMLP, an increase in stimulated superoxide anion and lysosomal enzyme release, and spontaneous shape change in these cells. The authors suggested the presence of trace amounts of the bacterial toxin lipopolysaccharide (LPS) in the Ficoll-Hypaque medium as the cause of this neutrophil priming (Haslett, 1985). This was confirmed by the absence of priming using an LPS free method of isolation, and the stimulation of quiescent cells by addition of LPS.
In addition the hypertonic conditions of Ficoll-Hypaque (Wells, 1977) may have primed the cells. In slightly hypertonic solutions cells lose water, thereby increasing their density. This principal has been used with some gradients, such as Nycoprep (Nycomed Pharma AS, Norway) for example, to allow the separation of similar sized lymphocytes and monocytes by their density. However, changes in the osmolality of the suspension medium may affect the cell (Chien, 1984; Dooley, 1981; Nash, 1988a). By contrast plasma/Percoll density gradients have a low osmolality and harvested cells appear functionally to be quiescent (Haslett, 1985).

Contaminating erythrocytes in the cell preparation are routinely removed by exposure to hypotonic solutions, such as ammonium chloride (NH_4Cl), but more commonly distilled water is used. Rinaldo and co-workers (1988) found the hypotonic lysis procedure was detrimental to leucocytes. They measured a 75% increase in the retention of neutrophils in the lungs following hypotonic lysis of erythrocytes, in comparison to cells not exposed to hypotonic lysis. These

neutrophils were, however, obtained by peritoneal lavage from rats and may have been functionally altered compared with peripheral blood neutrophils. However, assessment of superoxide anion production by the cells did not suggest priming (Rinaldo, 1988).

Although exposure to lower osmolalities can have adverse effects on the cell's ability to phagocytose and chemotact (Dooley, 1981), the exposure of neutrophils to either hypotonic saline solutions (0% - 0.8%) or NH_4Cl was found by several investigators to have no effect on cell function (Glasser, 1990; Haslett, 1985; Nash, 1988a).

The anti-coagulant used may also affect leucocyte function. Neutrophils in lithium heparin are easily activated, as determined by elastase release (Plow, 1982), compared to cells in citrate or EDTA (Chien, 1984). Indeed, addition of EDTA, a strong calcium chelator, to the cell suspension is often preferred to minimise such reactivity (Chien, 1984; Nash, 1988a). However, the presence of extracellular calcium may be important for intracellular signalling (Bengtsson, 1990; Berridge, 1989) and function (English, 1992) and is associated with the polymerisation and depolymerisation of the cytoskeleton (Janmey, 1987; Simchowitz, 1990). As previous studies suggest neutrophils from smokers are functionally activated or primed (Hoidal, 1982; Ludwig, 1982), neither EDTA or EGTA were considered suitable for use in the present studies. The removal of extracellular calcium in the smoke exposure studies could diminish any smoke-induced effect. Although citrate also acts by removing calcium, it is not as strong a chelator as EDTA or EGTA (Evans, 1984). Furthermore, cells in EDTA required a 50% increase in the initial suction pressure required for the cell to enter a micropipette compared with citrate or heparin treated cells (Evans, 1984). Although the low pH of citrate may adversely affect the leucocyte, as suggested by the increased cell adhesivity observed by Chien and associates (1984), Aoshiba and coworkers (1993) found no change in cell filtration for a range of pH.

The temperature at which the blood is stored before the leucocytes can be harvested, and the temperature at which the harvesting procedure takes place may also alter leucocyte function. Fearon and co-workers (1983) observed an increase in C3b receptors, which mediate immune and inflammatory reactions by attachment to soluble complexes or particles bearing C3b (the third component of the complement cascade), following exposure to temperatures above 20°C, which may affect cell function. Exposure to cold (4°C) did not alter neutrophil C3b receptor expression, phagocytosis and bacterial killing, nor prime neutrophils for activation. However, 1.5 hours at a low temperature (<4°C) severely reduced neutrophil locomotion and

chemotaxis (Chien, 1984; Glasser, 1990) and increased cell stiffness (Aoshiba, 1993). Relatively short term exposure to low temperatures could, however, be reversed by rewarming to 37°C (Aoshiba, 1993).

2.1.2 THE STUDY OF BLOOD CELL RHEOLOGY

Initially whole blood was studied as a bulk fluid, by measuring its resistance to flow i.e. its viscosity (Dintenfass, 1967; Fahraeus, 1929 & 1931). Any fluid under flow is subject to shearing, which is the sliding of parallel fluid layers over one another. Even a simple Newtonian fluid like plasma undergoes shearing when flowing in blood vessels (Lowe, 1987). A shear stress is the force applied to a fluid layer which produces its movement relative to an adjacent layer. Shear stress is greatest along the vessel wall where flow is slowest, and lowest in the centre where flow is fastest. The resistance to shearing, i.e. the ratio of shear stress to shear rate between the molecular and particulate components of each layer determines the fluid's viscosity. For Newtonian fluids, however, as the shear rate is hypothesised to be directly proportional to shear stress, the viscosity remains constant despite different shear conditions (Lowe, 1987). Since blood is a suspension of cells in plasma, it's viscosity is a function of plasma viscosity (Whittington, 1982). Plasma and serum viscosity are therefore routinely assessed in the clinical laboratory using capillary and tube viscometers, by manually applying a constant pressure head, or by commercially available "viscometers", using a centrifugal driving force.

Plasma viscosity is primarily determined by its protein composition and also by it's temperature. An increase in plasma viscosity is observed following the release of so called acute or chronic-phase proteins in response to trauma, surgery, infection or inflammation (Harkness, 1971). These large liver-derived plasma proteins (albumin, fibrinogen, α_1-macroglobulin, α_1-antitrypsin and the imunoglobulins) increase viscosity relative to their molecular size and asymmetry as they rotate during flow and displace water. Additionally, an increased plasma viscosity, due to an increase in large proteins particularly fibrinogen, can cause erythrocyte aggregation (Schmid-Schonbein, 1973), although with large increases in plasma viscosity, disaggregation of erythrocytes occurs (Schmid-Schonbein, 1968). The rouleaux formation of erythrocytes then disrupts the streamline flow of plasma, further increasing plasma and blood viscosity (Fahraeus, 1958). Erythrocyte aggregates also form in small vessels in low-flow states such as at vessel bifurcations and stenosis. Whole blood can not, therefore, be classified as a Newtonian fluid as its viscosity changes under different shear conditions.

In vessels with diameters <300 μm erythrocytes locate in the axial region where flow is fastest (Fahraeus, 1931; Schmid-Schonbein, 1980b & 1980c). By centring the erythrocytes in this central region of low shear forces, flow rates are enhanced under normal conditions (Fahraeus, 1931). Moreover, as the erythrocyte consists mainly of a fluid of low viscosity within a viscoelastic membrane (Schmid-Schonbein, 1981), the shear forces imposed upon the erythrocyte by plasma results in the deformation of the cell shape. The erythrocyte's intrinsic deformability, as well as being dependent on the cell's geometry (morphology and surface area to volume ratio), membrane flexibility and internal viscosity, is affected in suspensions by extrinsic factors such as shear conditions, haematocrit, the viscosity of the medium (plasma) and, when small, the vessel diameter (Lowe, 1981). Alignment of the elongated erythrocytes in parallel with the fluid streamlines encourages its participation in flow as it behaves not as rigid particles, but as elongated fluid droplets within blood (Dintenfass, 1962; Skalak, 1989). The extent of the shape change is greatly influenced by the magnitude of the external forces. The increased velocity of the erythrocytes in the axial stream effectively reduces the haematocrit. This phenomenon is known as the Fahraeus effect (1929). Moreover, the low dynamic haematocrit results in a lower blood viscosity as demonstrated by Fahraeus and Lindquist (the Fahraeus-Linquist effect)(1931).

ERYTHROCYTE RHEOLOGY

For microvessels with diameters below 20 μm the haematocrit is no longer the main determinant of blood viscosity and the flow behaviour of individual cells (microrheology) becomes more important. *In vivo* erythrocyte deformability has rheological importance as it allows the cell to reduce its diameter to traverse the narrow nutritive capillaries (Skalak, 1989), and conversely an increased rigidity of erythrocytes consequents their removal from the circulation by trapping in the spleen. Moreover, the influence of erythrocyte deformability on blood rheology is the cause of some pathological conditions. A reduced erythrocyte deformability is observed, for example, in sickle cell disease (Messer, 1970) due to the rigidity of the membrane; in spherocytic anaemias (Brereton, 1974) due to a reduced surface area to volume ratio; and in haemoglobin C disorders (Murphy, 1968) due to an increased internal viscosity as a result of the high haemoglobin content of the cell. Less severe changes in erythrocyte deformability have also been reported for diseases such as myocardial infarcts, diabetes, and vascular diseases (Dormandy, 1981; Juhan, 1982; Reid, Dormandy, 1976).

METHODS FOR MEASURING ERYTHROCYTE RHEOLOGY

There are presently several methods available for measuring erythrocyte deformability and new techniques are continually being devised (Greenaway, 1993; Hardeman, 1993). In general the techniques involve applying a force to the cell and measuring the amount of deformation produced by the force. The established methods include measurement of the viscosity of a washed erythrocyte suspension using viscometers (Stuart, 1985); the extent of shear deformation of erythrocytes adhered to a surface, using a flow channel (Fischer, 1978), or in suspension, using diffractometric methods (Bessis, 1975); the time or pressure required to aspirate erythrocytes into a micropipette (Evans, 1973); cell poking (Daily, 1984); or the filterability of cells through micropore membranes (Koutsouris, , 1985; Stoltz, 1984; Teitel, 1964; Thao-Chan, 1984; Zhu, 1989). The techniques for erythrocyte deformability have been reviewed by Chien (1977) and Lowe (1981).

LEUCOCYTE RHEOLOGY

The effect of leucocytes on blood rheology was initially ignored as they are few in number in comparison to erythrocytes and thus have a negligible effect on whole blood bulk viscosity and hence flow in large venules. However, the importance of leucocytes in small diameter vessels became evident by their influence on whole blood and erythrocyte filtration studies (Chien, 1983; Skalak, 1983). These findings were corroborated by *in vivo* observations of neutrophils plugging capillaries (Bagge, 1977; Lien, 1990; Warnke, 1992). As described in chapter 1, the physical characteristics of the neutrophil, in part, explains their prevalence for plugging filter pores *in vitro* and capillaries *in vivo*. These observations and their implications in disease processes has encouraged the study of leucocyte rheology.

Aspiration of cells into a glass micropipette using a suction pressure, and measuring their time of entry or the pressure required can be used to determine the deformability of individual cells (Evans, 1973; Frank, 1990a; Schmid-Schonbein, 1981). As whole or part cell aspiration can be performed, the influence of the cell membrane and the cell interior can be distinguished. Also different subpopulations can be identified. However, the micropipette technique is disadvantaged by being very time consuming.

Micropore filtration is similar to the micropipette as a population of cells in suspension pass through what can be viewed as a number of micropipettes together. The system is simple and therefore usable for routine clinical measurement of whole blood or erythrocyte "filterability". Boyden introduced the filter assay in 1962

(Boyden, 1962). Early studies were performed using paper (Teitel, 1964) or micropore filters with tortuous pores (Jandl, 1961). However, metal filters or polycarbonate membranes which contain cylindrical pores, introduced by Gregersen and associates (1967), are used predominantly today. These polycarbonate filters benefit by having a well defined geometry, with nearly cylindrical pores of uniform size. They are commercially available (Nuclepore, Costar Ltd., High Wycombe, UK) with pores of 3, 5 or 8 μm in diameter and approximately 10 μm to 11 μm in length. Although the pore length is shorter than the average systemic capillary (Weibel, 1984), they are comparable to the length of the capillary segments reported for the pulmonary microvasculature (Weibel, 1963) and are, therefore, ideal for modeling pulmonary blood flow *in vitro*.

Measurements of cell filtration are capable of detecting quite small changes in cellular rheology (Chien, 1983). As an end point, the resistance of a cell suspension to flow can be determined by measuring the pressure whilst keeping the flow rate constant (Chien, 1983), measuring the flow rate under a constant pressure (Lowe, 1981), the time to filter a volume (1 ml) (Aoshiba, 1993), or the volume filtered with time (Evans and Jones, 1992). The resistance of a suspension will be a function of the geometry of the filter (pore length, diameter and density), the viscosity of the suspension medium, and the behaviour of the cells as they enter, pass through, and leave the pores.

Recent advances with filtration systems have concentrated on measurement of the deformability of individual cells in suspension. Such instruments measure the transit time of cells through single pores under hydrostatic pressure. The Single Pore Rigidometer, as the name suggests, consists of only one pore (Frank, 1990a), whereas the Cell Transit Analyser contains a membrane with several pores (approximately 30) but the cell concentration and pressure applied are balanced such that generally only one cell passes through the membrane at any one time (Fisher, 1992; Zhu, 1989). Both instruments measure cell transit through the membrane by detecting a change in an electrical potential placed across the membrane as the cell enters a pore. These systems can be used to determine erythrocyte deformability without prior preparation to remove leucocytes as leucocyte transit times, being far longer than erythrocyte transit times, are rejected by the computer software (Zhu, 1989).

One problem with the filtration systems described above is the high shear stress to which the cells may be subjected. It is the pressure drop across the filter which creates the shear stresses applied to the cell causing it to deform, enter and traverse

the filter pores. The estimated shear stresses *in vivo* under normal conditions range from 5 to 50 $dyn.cm^{-2}$ (Atherton, 1972; House, 1989; Schmid-Schonbein, 1980b; von Adrian, 1992). Hence, as

$$\text{max wall shear stress in a filter} = \frac{\text{driving pressure x pore radius}}{2 \text{ x pore length}}$$

(Lowe, 1981; Reinhart, 1984), a maximum hydrostatic driving pressure of only 0.71 cm H_2O (714 $dyn.cm^{-2}$) can be applied for filters with 3 μm diameter pores and 0.46 cm H_2O (454 $dyn.cm^{-2}$) for filters with 5 μm diameter pores to maintain physiological conditions.

The most direct measurement of cell deformability was devised by Daily, Elson and Zahalak (1984). They employed a cell "poker" to indent the surface of the cell in a time-dependent manner, and measured both the force used and degree of indentation.

METHODS FOR MEASURING LEUCOCYTE RHEOLOGY

Several of the techniques developed for erythrocyte studies can be applied to investigate leucocyte deformability. Leucocyte deformability can be assessed using the effect of bulk shear stresses, as described for erythrocytes, however, the highly viscous leucocyte interior necessitates far greater shear stresses than are required with erythrocytes.

Observation of leucocyte passage through microcirculatory beds has provided information of the deformation characteristics of individual cells (Bagge, 1977; Lien, 1990; Warnke, 1992). However, micropipette aspiration and filtration through micropore membranes have been the key methods for the study of leucocyte deformation (Chien, 1983; Dong, 1988; Frank, 1990a; Gregersen, 1967; Nash, 1988b; Schmid-Schonbein, 1981). Micropipette studies revealed different deformation characteristics for the plasma membrane and the cell interior (Evans, 1989; Schmid-Schonbein, 1981). The micropipette technique can also be used to determine the deformability of different regions of the cell such as the pseudopods and the cell body of activated leucocytes (Skalak, 1984). Part cell deformation can also be assessed on adhered leucocytes using the cell "poker" (Daily, 1984; Peterson, 1982). However, micropipette aspiration or the 'poking' of single cells is limited by the small numbers of cells measured in each sample. Filtration systems, established for measurement of erythrocyte rheology, allow measurement of a large number of cells

as they traverse the pores in micropore filters, and have the additional advantage of their simplicity. These filtration methods are also attractive in that pore diameters can be chosen which mimic the diameter of capillaries, hence cell deformability can be determined under circumstances analogous to those existing in the microcirculation. Moreover, filtration systems have been developed, such as the Cell Transit Analyser (CTA)(Zhu, 1989), which can assess deformability of a large number of individual cells, but in a short period of time.

FILTRATION SYSTEMS

The filtration of a leucocyte suspension through polycarbonate filters of fixed pore number and size, is usually expressed relative to the filtration of cell-free buffer. This, in effect, calibrates each filter. The pressure/time curves (Figure 2.1) developed by cell suspensions have been described by several authors (Chien, 1983; Nash, 1990; Reinhart, 1987; Schmalzer, 1983). Relative to the pressure developed by buffer alone, the initial pressure (P_i) produced by filtration of a cell suspension (for example of leucocytes) is observed within a few seconds. This first phase is thought to reflect the arrival of cells at the filter and thus relates, in a mixed population, to the numerically predominant cell type in the suspension (neutrophils). The slope of this initial increase in pressure is also influenced by the ratio of cell number to the number of pores (Chien, 1983; Nash, 1990). The second phase of the curve, which develops at a more gentle rate, is influenced by a less deformable cell population in the sample (monocytes)(Chien, 1983; Nash, 1990). Although smaller in number, these cells are more resistant to flow through the pores and thus produce a greater pressure. As these cells dislodge from the pores the fast pressure increase seen in the initial phase of filtration is prevented, and the development of a plateau pressure is observed. Pore blocking, however, results in a sustained pressure rise (Chien, 1983; Nash, 1990). An analogous curve would be created by monitoring changes in flow in a constant pressure filtration system.

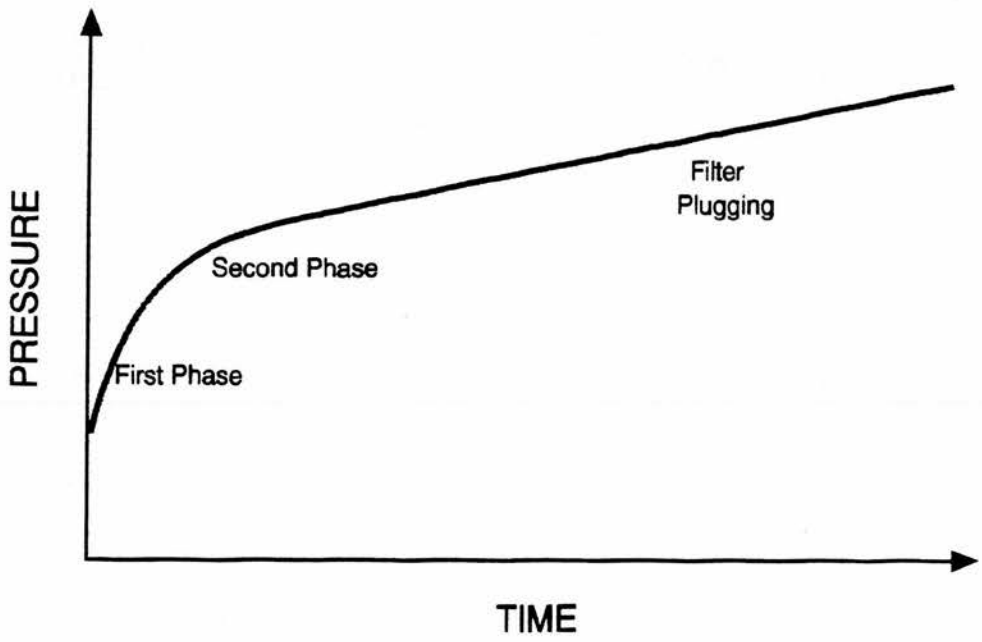


FIGURE 2.1
Schematic illustration of a pressure time curve representing the filtration of a cell suspension through a micropore membrane at constant flow. The phases of the curve indicated are described in the text.

Theoretical calculations (Chien, 1983; Schmalzer, 1983) and modelling (Skalak, 1983) of pressure or flow/time curves provides information about the different cell populations in a mixed cell suspension, such as their relative proportions, resistance and pore transit time. From studies such as these, the influence of contaminating leucocytes during the assessment of erythrocyte deformability was highlighted (Chien, 1983; Schmalzer, 1983). By theoretical analysis of pressure/time curves obtained from the filtration of suspensions of leucocytes and erythrocytes in mixed proportions, Chien and associates (1983) calculated that to produce an equivalent initial fast phase of filtration, which is influenced by cell concentration, 700 erythrocytes would be required for every one neutrophil. It is of interest, therefore, to note that the normal concentration of erythrocytes in blood ($5x10^{12}$ RBC.l^{-1}) is approximately 700 times that of the normal neutrophil concentration (7×10^9 PMN.l^{-1}).

Calculations by Chien et al (1983) on the second phase of the curve with it's slower pressure rise, which as discussed above is more representative of whole cell deformability, revealed a ratio of 1000 erythrocytes to one neutrophil was required to produce a similar rate of increase. That the cellular viscosity of neutrophils (130 ± 5 dynes.s^{-1}.cm^{-2})(Schmid-Schonbein, 1981) is approximately 1000 times the viscosity of erythrocytes (0.1 - 0.6 dynes.s^{-1}.cm^{-2})(Chien, 1981; Cokelet, 1968) suggests dominance of the viscoelastic property of the neutrophil's cytoplasm over the plasma membrane as regards the cell's deformability. These data highlight the important influence of neutrophil deformability on the circulation of blood through microvascular beds.

2.1.3 MODELS OF NEUTROPHIL MECHANICAL BEHAVIOUR

Deformation of the erythrocyte, as already mentioned, is influenced by the geometry of the cell, the viscosity of the haemoglobin solution, and the viscoelasticity of the membrane (Chien, 1981; Schmid-Schonbein 1981). The time-dependent deformation of the erythrocyte, upon the application of a pressure, and it's recoil upon release can be described by the Kelvin model of an elastic element in parallel with a viscous element (Chien, 1978). The model describes the viscoelastic properties of the plasma membrane and associated underlying spectrin cytoskeleton. The cytoplasmic haemoglobin solution (a Newtonian fluid) of the erythrocyte interior has little viscous properties and no elasticity and thus provides minimal resistance to deformation (Tozeren, 1984).

The neutrophil interior, by contrast has high viscosity and significant elasticity (Braide, 1991; Chien, 1984) which must be incorporated in to the model. Several models have been proposed to describe the mechanical behaviour of quiescent neutrophils (Bagge, 1976; Evans, 1984; Schmid-Schonbein, 1981). Naturally for any modelling of complex systems, much simplification occurs and assumptions are made. For the models of neutrophil deformation, the volume was assumed to remain constant.

The first model developed represented the neutrophil as a **viscoelastic solid sphere**, based on observations of small deformations in the first few seconds of micropipette aspirations (Bagge, 1976; Schmid-Schonbein, 1981). The model consists of three elements - a Maxwell element, which is composed of a viscous element (μ) in series with an elastic element (k_2), with an elastic spring (k_1) in parallel (Figure 2.2)(Bagge, 1976; Schmid-Schonbein, 1981). In the model the Maxwell element represents the cell interior with the elastic element representing the cell membrane and thus the restoring forces (Bagge, 1976).

Aspiration of a cell when a pressure is applied results in immediate elastic deformation which then continues more slowly, and is accounted for in the model by the Maxwell element. After a long period of time the solid model assumes that, due to the elastic stiffness (k_1), eventually no further deformation occurs (Schmid-Schonbein, 1981). However, experimental observations found that once a force in excess of the threshold pressure (an initial pressure resulting from membrane tension which has to be overcome before such deformation can occur) was applied and maintained, continuous deformation of the neutrophil resulted (Evans, 1984; Yeung, 1989). Moreover, the model parameters obtained for small deformations (Schmid-Schonbein, 1981), compared with the parameters obtained for large deformations (Evans, 1989) of cells in a micropipette were discrepant. For example, the model predicted a 20-fold lower viscosity (μ) for small deformations of 1-2 seconds (130 $dyn.s^{-1}.cm^{-2}$)(Schmid-Schonbein, 1981) than for longer deformations of 10-20 seconds (2.1×10^3 $dyn.s^{-1}.cm^{-2}$)(Evans and Yeung, 1989). Thus, as recognised by Schmid-Schonbein (1981), although adequately describing small deformations, the viscoelastic solid model is inadequate for large deformations and longer time periods. A second model for passive neutrophil deformation was therefore introduced by Evans and Kukan (1984).

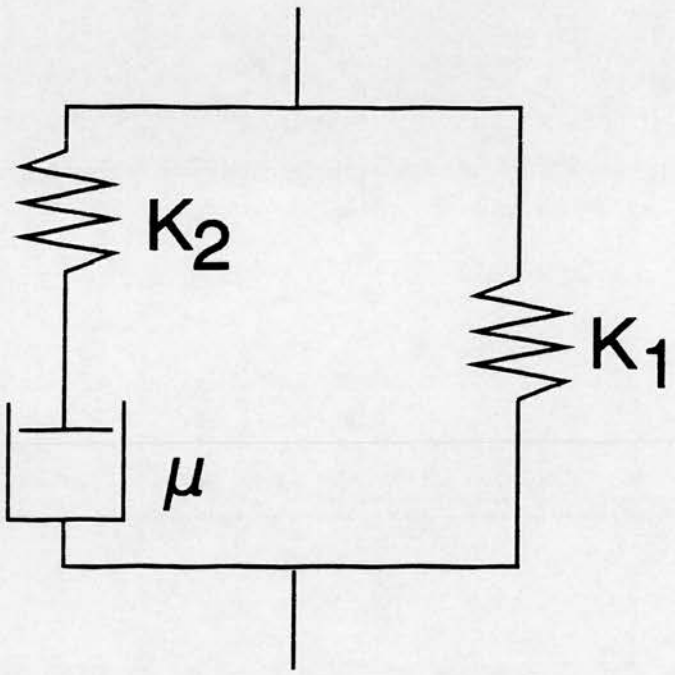


FIGURE 2.2
Viscoelastic solid model of passive neutrophil deformation. The model comprises a Maxwell element composed of a viscous element (μ) in series with an elastic element (k_2) with an elastic spring (k_1) in parallel (Schmid-Schonbein, 1981).

To fit their experimental data, Evans and Kukan (1984) proposed a structural model comprising a "contractile surface carpet" surrounding a liquid interior. By disturbing the integrity of the cytoskeletal network in the cortical cytoplasm, by addition of colchicine or cytochalasin B which cause depolymerisation of the microtubules and actin filaments respectively, the viscoelasticity and flow resistance of the neutrophil was reduced (Chien, 1985). Hence the surface tension of the cell appears to be created by the cortical cytoskeleton adjacent to the plasma membrane, with the ruffled membrane of the neutrophil, unlike that of the erythrocyte, adding little resistance (Evans, 1989). Although, in a situation where all the excess membrane has been used up, for example by osmotic swelling of the cell, the membrane would become very resistant to changes in surface area (Chien, Schmid-Schonbein, 1984; Nash, 1988a). The **liquid drop model** (Yeung, 1989) therefore consists of a Newtonian liquid, to describe the viscous-liquid nature of the cell interior (represented by the Maxwell element), surrounded by a persistent tension (represented by an elastic spring).

The recovery of large deformations is also inadequately described by the viscoelastic solid model. The recovery times experimentally observed following large deformation (Sung, 1988) and predicted by the liquid drop model (Evans, 1989) were slower than that predicted by the viscoelastic solid model (Schmid-Schonbein, 1981). Leucocytes recover to their spherical state after deformation with an initial elastic, followed by a gradual viscoelastic recoil (Bagge, 1976). As recovery from deformation was essentially linear with respect to time (Evans, 1984), it would appear to be driven by surface tension (k_1, Figure 2.2), likely the cortical cytoskeleton of the cell, with the viscous liquid interior resisting recovery (Evans, 1984; Schmid-Schonbein, 1981). As the interior of the cell has a greater viscosity than suggested by measurement of small deformations by micropipette aspiration and predicted by the viscoelastic solid model (Schmid-Schonbein, 1981), there is also greater resistance to recovery which results in slower recovery times.

However, the purely viscous Newtonian fluid interior of the liquid drop model does not allow for the immediate deformation of the cell observed when pressure is applied, or the initial elastic response of the cell when pressure is released, Dong and associates (1988) therefore proposed a "**Maxwell liquid drop**" model to represent large deformation of the neutrophil. A Maxwell fluid, describing the interior, accommodates both the viscoelastic properties of the cytoplasm and granules and the large nucleus of the neutrophil which are not taken into consideration in Evans's model (1984).

Alternatively, Skalak and associates (1990) have proposed a three layer model. They speculated that neutrophil mechanical deformation can be best represented by an outer cortical shell, with a Newtonian fluid of low viscosity as the second layer and a highly viscous core describing the nucleus and its cytoskeleton. As before, the outer shell would represent the cortical tension. For rapid small deformations, the low viscosity of the middle layer would be the main determinant, and for the longer, large and slow deformations, the inner core would dominate (Skalak, 1990).
Micropipette aspiration of part of the cell, and likewise measuring the deformation of only part of the cell using the cell poker, can be described by the viscoelastic solid model. Cell filtration through micropore membranes involving whole cell deformation under pressure is, however, best described by the liquid drop method, or more comprehensively, the Maxwell liquid drop.

2.1.4 *IN VITRO* CIGARETTE SMOKE EXPOSURE

The association between exposure to tobacco smoke and chronic respiratory disease has initiated many studies on the effect of cigarette smoke on lung cell biology and biochemistry. Measurements comparing chronic smokers with lifelong non-smokers have revealed changes in anti-oxidant levels (Calder, 1963; Galdston, 1984; Toth, 1986) and the concentrations (Hunninghake, 1983) and function (Corberand, 1979; Hoidal, 1981) of leucocytes in both the circulation and in the alveolar space. Studies have also been undertaken in animal models to overcome the problem of the large individual variations which occur in human studies (Bosken, 1991; Doerschuk, 1988b; Gillespie, 1987; Janoff, 1979b; Minty, 1985; Smith, 1986). The animal studies have the additional advantage in that the exact time sequence of the inflammatory process in the lungs induced by cigarette smoke can be more precisely defined. Furthermore, *in vitro* studies on isolated cell types allow investigation of the effect of cigarette smoke at the cellular level (Bridges, 1977; Corberand, 1980; Moldeus, 1985; Smith, 1986; Voisin, 1985).

Various forms of cigarette smoke have been employed to study short term effects *in vivo* or *in vitro* depending on the model under investigation. For animal studies, isolated lung preparations, and *in vitro* work with isolated cells (in confluent culture or suspended in an appropriate medium), direct exposure to either whole or vapour phase cigarette smoke, cigarette smoke condensate (CSC) or aqueous extracts of cigarette smoke can be used.

Aqueous smoke extracts are obtained by bubbling the fresh smoke from several cigarettes through cell-free culture media which can then be added to the cells (Bridges, 1977). CSC is obtained by condensation of whole smoke onto the sides of a glass vessel which is then washed off by an appropriate solvent such as dimethylsulfoxide (DMSO)(Moldeus, 1985).
For direct exposure of animals or cell culture to fresh smoke, an exposure chamber can be entirely filled with smoke (Powell, 1971) or smoke may be pulsed into the chamber (Holt, 1973). A cell monolayer would, preferentially, be covered by a minimal amount of medium. Voisin and colleagues (1977) developed a rocker system to allow direct exposure of a confluent cell layer to fresh cigarette smoke without even a minimum nutrient medium covering. The rocker system, however, ensured an intermittent covering with medium to prevent the cells from drying out.

The system of smoke exposure developed for the present studies generates vapour phase cigarette smoke by a smoking machine which is "puffed" at minute intervals over a cell suspension held in a siliconised glass tonometer (shown in Figure 2.4). The tonometer, maintained at 37°C by a waterbath, is gently rotated to produce a thin layer of the cell suspension. The surface area of the cell suspension is thereby increased, exposing a greater number of cells to the cigarette smoke. Also under these conditions cells are surrounded by little fluid and hence exposure is more analogous to the *in vivo* situation within the pulmonary capillaries.

The chemical composition of cigarette smoke is highly complex, consisting of a myriad of volatile compounds and particulate matter (section 1.7.3)(Dalhamn, 1968; Heseltine, 1987; Jenkins, 1984; Richards, 1992). Moreover, the synergistic effect of smoke components has been highlighted (section 1.7.3)(Church, 1985; Richards, 1992).
To test the toxicity of each individual smoke component would be a major task. However, cigarette smoke is frequently investigated as two major fractions: whole particulate and vapour phase cigarette smoke (Borland, 1985; Bridges, 1977; Rylander, 1974). The presence of filters in the cigarette removes some of the particulate matter. The remainder can be removed by passing cigarette smoke through a Cambridge filter. The filtering properties of the nasal and oral cavities is reported to remove a proportion of the water-soluble toxic substances of smoke, and approximately 50% of the carbon monoxide (Dalhamn, 1968). However, some water-soluble and a high proportion of water insoluble fractions of cigarette smoke and particulate matter are retained deep in the lungs (Dalhamn, 1968). It is probable

that these volatile substances are bound to the particulate matter, as the removal of particulate components of smoke by a Cambridge filter is observed to reduce the presence of these volatile components in the lungs (Dalhamn, 1966; Eichel, 1969).

Various methods allow routine assessment of smoke intake and thus smoking status. Cotinine, the major metabolite of nicotine is the preferred marker because of its sensitivity and specificity, whereas thiocyanate, for example, is influenced by diet, and carboxyhaemoglobin (COHb) is influenced by enviromental carbon monoxide (Barlow, 1987). However, cotinine determinations are performed on high performance liquid chromatography (HPLC) or by radioimmunoassay (Aldkofer, 1989) which are not suitable for routine analysis. The measurement of COHb levels in exposed whole blood samples was used to indicate the level of cigarette smoke exposure *in vitro* in these studies as it is simple. Any influence of enviromental carbon monoxide would be minimal in comparison to the dose of smoke.

2.2 AIMS

To isolate pure neutrophil populations from peripheral whole blood and demonstrate their functional quiescence and integrity.

To validate a constant flow filtration system for assessment of the filterability of cell suspensions, as a measure of cell deformability.

To establish a system which will allow reproducible exposure of cell suspensions to vapour phase cigarette smoke *in vitro*.

2.3 MATERIALS AND METHODS

REAGENTS

All reagents were obtained from Sigma Chemical Company (Poole, UK) unless stated otherwise. Phosphate buffered saline (PBS) contained 145 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (pH 7.4, 290 $mOsm.kg^{-1}$). Stock solutions of phorbol myristate acetate (PMA) in dimethyl sulfoxide (DMSO); n-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) in PBS; and horseradish peroxidase in 0.05 M phosphate buffer were prepared and stored at -20°C. Superoxide dismutase (SOD) and phenol red were dissolved in distilled water and stored at -20°C and 4°C respectively. Albumin (Boehringer, Mannheim, W Germany) and cytochrome C were made up fresh, before use, with PBS.

2.3.1 NEUTROPHIL HARVESTING

The entire harvesting procedure was carried out in a Laminar flow hood (Howorth Air Engineering LTD, Bolton, UK) at ambient temperature immediately following blood sampling. All reagents and containers used for the isolation procedure were endotoxin free according to the manufacturers specification or tested by the Limulus Polyphemus Amoebocyte Lysate assay (E-toxicate, Sigma, Poole UK) to contain undetectable levels (<0.005 $ng.ml^{-1}$). Peripheral venous blood, anti-coagulated with acid trisodium citrate-D-glucose (ACD: Blood Transfusion Service, National Protein Fractionation Unit, Edinburgh)(pH Eur Formula 1A: 2.2, 0.8, and 2.45 $g.ml^{-1}$ respectively)(1 ml ACD/ 7.5 ml blood), was obtained from healthy normal, non-smoking individuals by venepuncture using a 19G needle. Fifteen millilitres of the blood was mixed with 6% Dextran 70 (70,000 mw, Travenol laboratories, Norfolk, UK) and left for approximately 1 hour to allow sedimentation of the erythrocytes. Autologous plasma was prepared from the remaining 15 mls of blood by centrifugation for 10 minutes at 1200 rpm to separate out the plasma which was then centrifuged for a further 20 minutes at 3000 rpm.

A discontinuous density gradient was prepared by adjusting 90% Percoll (Pharmacia, Uppsala, Sweden: diluted with 9% saline) to densities of 42% and 65% Percoll by the addition of autologous plasma. The gradient was constructed by carefully overlaying 2 ml volumes of the lower density (42%) over the higher density (65%) in a 15 ml conical tube.

Leucocyte rich plasma was obtained from the dextran sedimented blood sample and washed once with PBS. The resulting cell pellet was resuspended in 2 mls of autologous plasma and layered on top of the pre-prepared discontinuous

plasma/Percoll gradient which was centrifuged at 1100 rpm for 10 minutes to density separate the cells. The neutrophil population was obtained from a second band, at the interface of the two densities. Following a further wash in PBS, contaminating erythrocytes were lysed by the addition of 0.2% saline for 30 seconds followed by the return to normal isotonicity by the further addition of an equal volume of 1.6% saline. After centrifugation at 1100 rpm, the resulting neutrophil pellet was resuspended in PBS to which 0.5% bovine serum albumin was added (PBS/BSA: pH 7.4) unless stated otherwise. A manual cell count was performed using a haemocytometer and Olympus light microscope (BHT System, Olympus Optical Co. Ltd., London, UK) on an aliquot of the cells stained with crystal violet in 1% acetic acid which also allowed assessment of the purity of the cell suspension. Random samples were assessed for viability and erythrocyte contamination by staining with trypan blue or $Na_2C_6H_6O_7.2H_2O$ ($32g.l^{-1}$) with 10 ml 40% formaldehyde added respectively and performing a manual count as above.

2.3.2 ASSESSMENT OF NEUTROPHIL FUNCTION

CELL MORPHOLOGY

Cell morphology was assessed by light and transmission electron microscopy for changes in cell shape as evidence of cell activation following harvesting (Haslett, 1985). For transmission electron microscopy (TEM) the cells were washed once and fixed by resuspension in fresh gluteraldehyde and stored at 4 °C until they could be processed.

For TEM preparation, the neutrophils were placed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hour, dehydrated through 10, 50 and 100% absolute alcohol in PBS for 15 minutes each. The cells were then placed in propylene oxide twice for 10 minutes, and impregnated in Araldite resin overnight. Each sample was further embedded in Araldite resin and polymerised at 50 °C before being cut on a LKB Ultrotome Nova (thickness 60 nm), and stained with uranyl acetate and lead citrate. The cells were viewed and photographed from the TEMs using a Jeol Jem-100s electron microscope at random.

HYDROGEN PEROXIDE ASSAY

Hydrogen peroxide (H_2O_2) generation was measured by the method of Pick and Keisari (1980). The reaction mixture consisted of 100 ml 10 mM potassium phosphate buffer containing 1 ml of horseradish peroxidase ($5\ mg.ml^{-1}$) in 0.05 M potassium phosphate buffer; 1 ml of 0.028 M phenol red; 140 mM sodium chloride and 5.5 mM dextrose. Neutrophils (2.5×10^5 cells) were added to 1 ml of the

reaction mixture. Both the spontaneous and PMA stimulated (0.1 and 1 $\mu g.ml^{-1}$) release of H_2O_2 was measured for 2 hours after incubation at 37°C in 5% CO_2. The reaction was terminated by centrifugation at 2000 rpm at 4° C for 10 minutes. The colour reaction was completed by alkalisation with 1N NaOH. The absorbance of the supernatant fluid was measured on a Pye unicam SP8-400 spectrophotometer (Unicam Ltd, Cambridge, UK) at 610 nM and compared with a standard curve derived from dilutions of a reference solution of H_2O_2.

SUPEROXIDE ANION ASSAY

Superoxide anion (O_2^-) generation was measured as the superoxide dismutase (SOD) -inhibitable reduction of cytochrome C (Johnston, 1981). The reaction mixture contained 80 μM cytochrome C and 0.65 mM dextrose in 50 ml of PBS. Tubes containing 1ml of the reaction mixture and 2.5×10^5 neutrophils were set up with identical tubes which, in addition, contained 375 units SOD. Release of O_2^- either spontaneously, or following stimulation with 0.1 and 1 $\mu g.ml^{-1}$ PMA, was measured after incubation for 2 hours at 37° C in 5% CO_2. The reaction was terminated by centrifugation at 2000 rpm at 4° C for 10 minutes. The difference in absorbance of the supernatant fluids, in the presence or absence of SOD, was determined in a Pye Unicam SP8-400 spectrophotometer at 550 nm. The amount of reduced ferricytochrome C was calculated based on an extinction coefficient of 21.0 $mM.cm^{-1}$ for cytochrome C.

ELASTASE RELEASE

Immunoreactive elastase was measured by Dr I McGregor, Blood Transfusion Centre, Edinburgh, in supernatants obtained from control neutrophil suspensions (2.5×10^5 $cells.ml^{-1}$) and following stimulation with 1 $\mu g.ml^{-1}$ PMA for 10 minutes at 23°C, by the method of Plow (1982). In brief, quantification of neutrophil elastase was performed using a competitive-inhibition radioimmunoassay (RIA) of the double antibody type. Elastase was isolated from human leucocytes using ion exchange and affinity chromatography, and iodinated with ^{125}Iodine. One ml of test plasma or standard was added to 0.25 ml of ^{125}I-elastase (15 $ng.ml^{-1}$ in 0.6M sodium chloride/ 0.05M sodium borate) followed by addition of antiserum to leucocyte elastase prepared from rabbit serum. Competition between the elastase in the test sample and the radio-iodinated elastase for the antibody allowed the elastase levels in the sample to be determined from standard curves of ^{125}I-elastase and known serial dilutions of standard. Both free elastase and enzyme-complexed elastase were measured by this RIA.

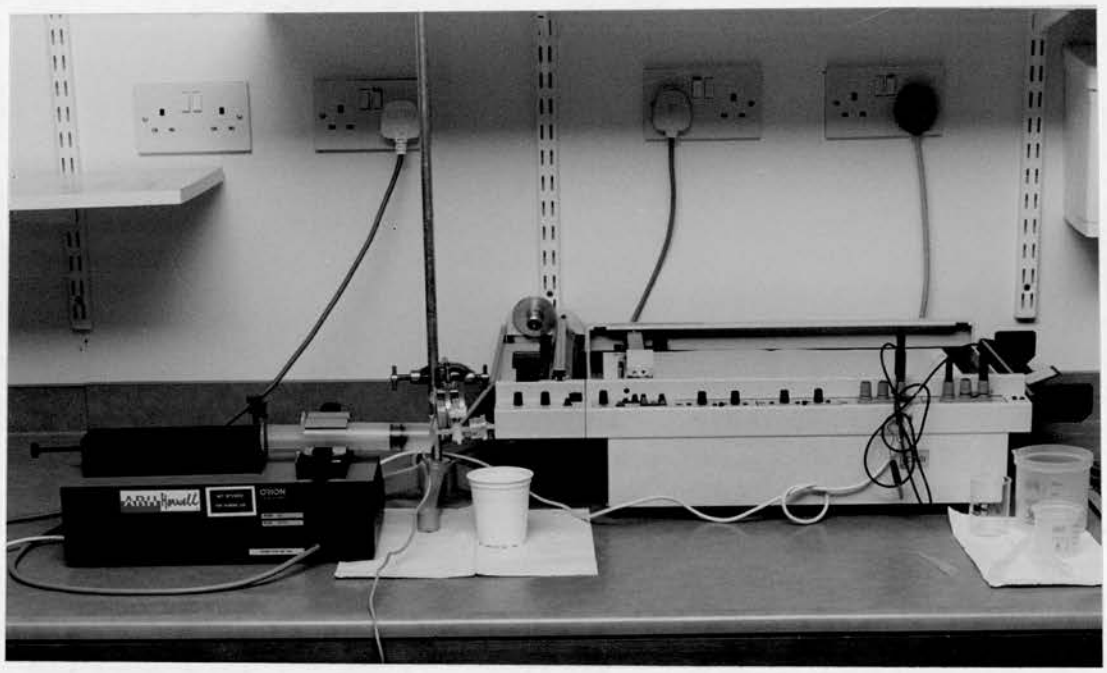
Neutrophil filterability was measured by the method of Lennie and colleagues (1987). The cell suspensions were prepared by suspending the cells in PBS containing 0.5% BSA. The buffer (PBS/BSA) alone or cell suspensions were placed in a 30 ml syringe and infused using a syringe pump (Orion, model 351) at a constant flow rate of 1.5 $ml.min^{-1}$ through 13 mm polycarbonate membranes with pore dimensions of 5 μm diameter, 11 μm length (Nuclepore) housed in a 'pop-top' filter holder. An in-line quartz pressure transducer (Hewlett Packard, model 1290A) and amplifier (Hewlett Packard, model 78353A) were used to measure the pressure developed over 6 minutes of filtration which was recorded on an XY recorder (Watanabe, Model WX4301)(Figures 2.3a and b). The pressure recorder was calibrated between 0 and 21 cm H_2O using a water manometer. Before the filtration of a cell suspension, each filter was calibrated to establish the baseline pressure (P_0) by filtration of buffer alone. The pressure developed by a cell suspension was calculated as the absolute pressure above baseline.

To determine the optimum cell concentration for the filtration studies, neutrophil suspensions at several concentrations (2×10^4 ml^{-1}, 5×10^4 ml^{-1}, 1×10^5 ml^{-1}, and 5×10^5 ml^{-1}) were filtered as described above.

To assess reproducibility of the filtration system, 2 or 3 replicate filtrations, at a concentration of 1×10^5 ml^{-1}, were performed in succession on neutrophil aliquots from 5 subjects.

The effect of time on neutrophil filterability was investigated by filtering neutrophils ($1 \times 10^5 ml^{-1}$) immediately following isolation of the cells, after 40 minutes, 1 hour and 3.5 hours on three occasions.

To investigate the effect of activation on neutrophil filterability, neutrophils ($10^5.ml^{-1}$) were stimulated by 10 minutes incubation with 5 $\mu g.ml^{-1}$ PMA, or the addition of 10^{-7}M fMLP immediately prior to filtration.



FIGURES 2.3a and b
Photograph (a) and illustration (b) (see following page) of the constant flow filtration system used to measure the 'filterability' of a cell suspension. Cell filtration has been shown to reflect cell deformability, as measured directly by the cell poker technique (Worthen, 1989).

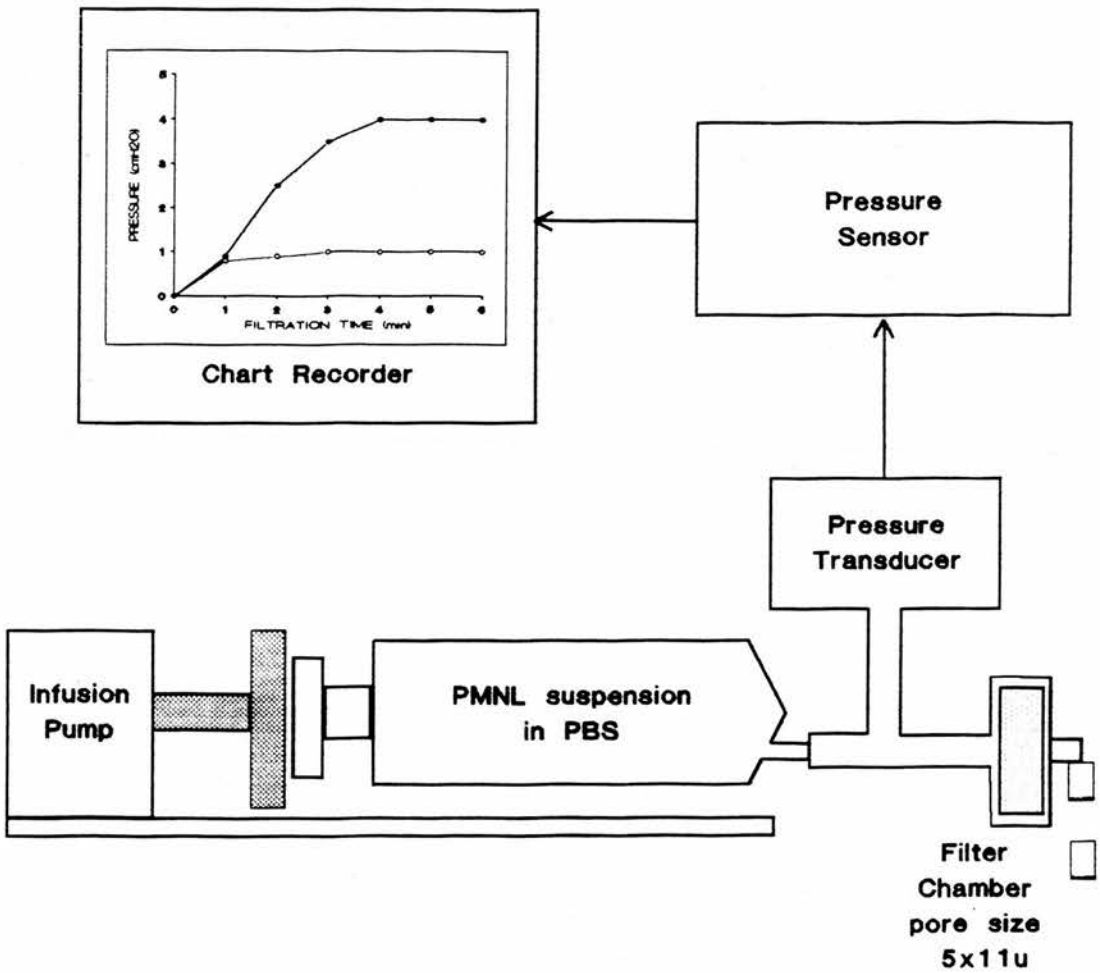


FIGURE 2.3b

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2.3.4 *IN VITRO* CIGARETTE SMOKE EXPOSURE SYSTEM

Cell suspensions (2 ml) were placed in a siliconised glass tonometer, maintained at 37°C in a water bath and gently rotated for 4 minutes (Figure 2.4). The smoke from a standard medium tar cigarette was drawn through a Cambridge filter to filter out the particulate content, and delivered across the cell suspensions in 35 ml puffs of 2 seconds duration, once every minute. Control samples were sham exposed in air. The smoke generating machine, cigarettes, and Cambridge filters were kindly provided by WD and HO Wills, Imperial Tobacco LTD, Bristol, UK.

To establish the dose of cigarette smoke delivered to the neutrophil suspensions in the tonometer, whole blood samples were exposed in a similar manner to 3, 5 or 7 puffs of cigarette smoke, and the level of COHb measured using a co-oximeter (model 282, Instrumentation Laboratory, Lexington, USA).

To allow reproducible smoke exposure of the cell suspensions placed in the tonometer, a schedule was established for smoke exposure and machine clearance. Following exposure of cell suspensions to vapour phase cigarette smoke, the machine was cleared by drawing through a standard number of puffs of air. The COHb levels in samples of whole blood exposed to 2 or 4 puffs of cigarette smoke, following such a schedule, were measured to determine consistency of the dose of smoke delivered by the smoking machine.

The effect of of cigarette smoke exposure (5 puffs) on pH levels of buffer and whole blood was also determined.

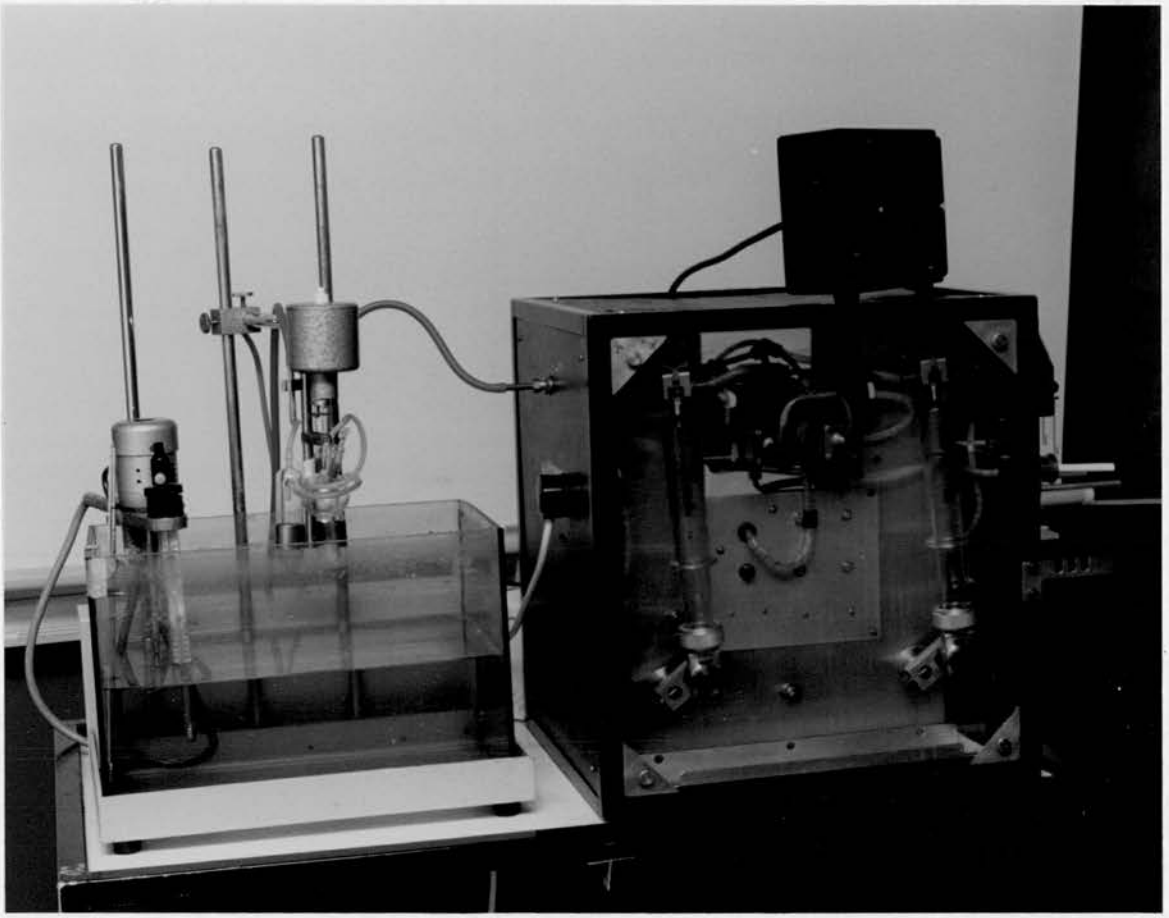


FIGURE 2.4
The system used to expose cell suspensions to cigarette smoke *in vitro*. Comprising a smoke generating machine and a siliconised glass tonometer, to hold the cell suspensions, which was maintained at 37°C by a waterbath.

2.3.5 STATISTICAL ANALYSIS

All statistics analysis in this thesis, unless stated otherwise, were performed using the SPSS statistics package for IBM personal computers. Advice as to the statistical test most appropriate for the each type of experiment was gratefully received from Dr's PK Wraith and K Donaldson.

For comparison of paired samples, differences between means of treatment groups were tested using a students t-test. For data where multiple measures were to be compared, a oneway analysis of variance (ANOVA) with Scheffe F-test corrections for multiple comparisons was employed (Snedecor, 1974).

2.4 RESULTS

2.4.1 NEUTROPHIL HARVESTING

The reagents used in the neutrophil harvesting technique, employed in these studies, were tested using the limulus amoebocyte lysate assay and found to be endotoxin free (Table 2.1).

Using the harvesting technique described, a mean recovery $1.14 \pm 0.6 \times 10^7$ neutrophils (23.5%: n=10) was obtained from 15 mls of whole blood. The neutrophil suspensions were 96.9 ± 2.2% pure (n=10) and <2% erythrocytes were present. The neutrophils were assessed to be >98% viable by trypan blue exclusion.

2.4.2 FUNCTIONAL ASSESSMENT OF HARVESTED NEUTROPHILS

CELL MORPHOLOGY

No cell aggregate formation, shape change or degranulation was apparent under light or electron microscopy (Figure 2.5) to suggest activation of freshly isolated neutrophils.

PRODUCTION OF REACTIVE OXYGEN INTERMEDIATES AND PROTEASE RELEASE

Low levels of H_2O_2 and O_2^- were produced spontaneously by harvested neutrophils, and production of these radicals was significantly enhanced by PMA stimulation (Table 2.2).

Low levels of immunoreactive elastase were measured for spontaneous release from harvested neutrophils (4.7 ± 4.0 ng.ml^{-1}), which were elevated following PMA stimulation (12.2 ± 8.0 ng.ml^{-1}; n=6, $p<0.01$).

2.4.3 NEUTROPHIL FILTRATION

EFFECT OF CELL CONCENTRATION ON FILTRATION PRESSURES

The filtration of neutrophil suspensions, over 6 minutes, developed higher pressures with increasing cell concentrations (Figure 2.6).

REPRODUCIBILITY OF NEUTROPHIL FILTRATION.

The intra-individual reproducibility of neutrophil filtration (cell concentration 1×10^5.ml^{-1}) using a constant flow filtration system was demonstrated for 5 subjects (Figure 2.7).

THE EFFECT OF TIME ON THE MEASUREMENT OF NEUTROPHIL DEFORMABILITY.
The filtration pressures developed by neutrophils were unaltered within 1 hour, but were increased after 3.5 hours following cell harvesting (Figure 2.8).

THE FILTRATION OF STIMULATED NEUTROPHILS.
Neutrophils stimulated by the addition of PMA developed significantly higher filtration pressures compared with control neutrophils (Figure 2.9a). The pressures increased to a plateau after about 4 minutes filtration. Similarly stimulation with fMLP consistently increased filtration pressures which, however, peaked after 3 minutes filtration (P_3) and then fell again (Figure 2.9b).

The rate of the initial pressure rise, calculated as the gradient (P_{grad}), measured a more rapid increase in filtration pressures following fMLP stimulation (P_{grad} 14.9 $cm.H_2O.cm^{-1}$) than following PMA stimulation (P_{grad} 5.4 $cm.H_2O.cm^{-1}$).

2.4.4 *IN VITRO* SMOKE EXPOSURE

The COHb levels measured in 2 ml whole blood samples following exposure to 3, 5 or 7 puffs of vapour phase cigarette smoke in the tonometer system are listed in Table 2.3. The COHb levels increased with increasing dose of smoke exposure.

REPRODUCIBILITY OF THE SMOKE EXPOSURE SYSTEM.
Table 2.4 lists the %COHb levels measured in 2 ml aliquots of whole blood exposed to 2 or 4 puffs of vapour phase cigarette smoke on 3 separate occasions. By following the schedule of clearance of the smoke generating machine (7 puffs) between each cigarette, a reproducible dose of smoke was delivered to cell suspensions in the tonometer.

THE EFFECT OF *IN VITRO* SMOKE EXPOSURE ON BLOOD AND BUFFER PH LEVELS.
The pH levels in PBS/BSA were reduced, whereas pH levels in samples of whole blood were increased following exposure to either air or smoke (Table 2.5).

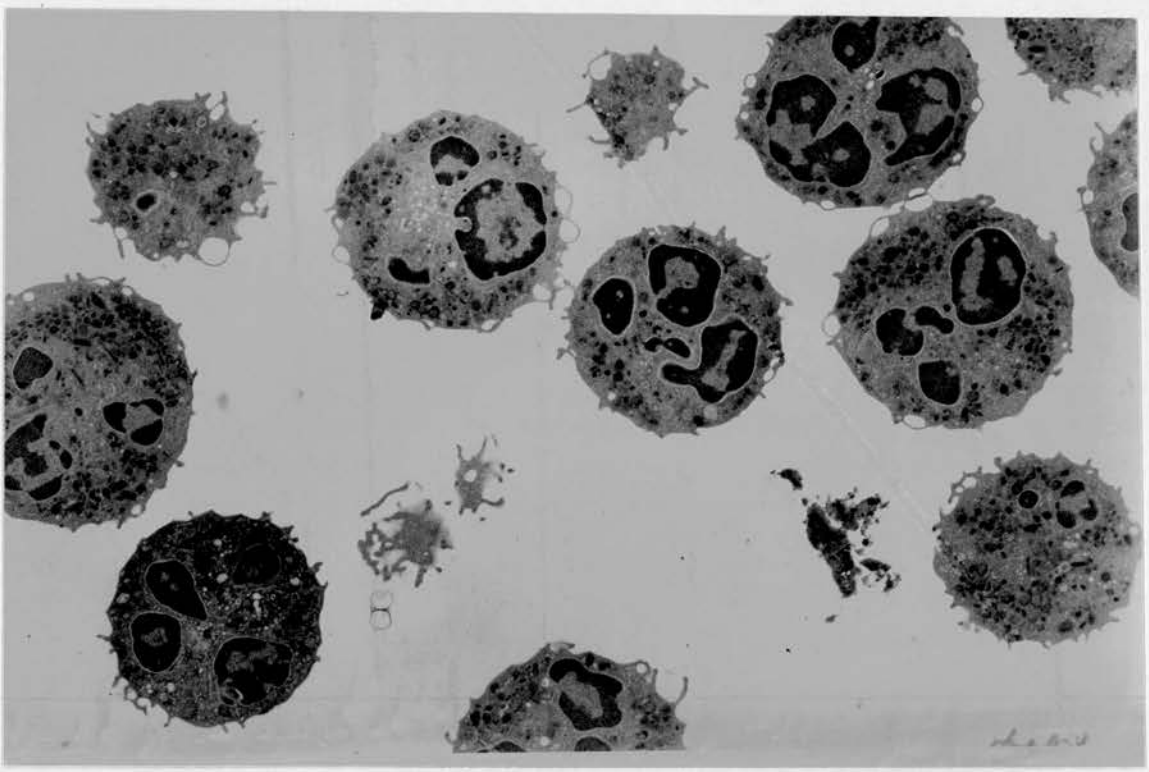


FIGURE 2.5
Transmission electron micrograph (x4.2 x 10^3 magnification) of quiescent neutrophils isolated from whole blood using a plasma/Percoll density gradient. Single neutrophils are shown with round morphology and intact cytoplasmic granules.

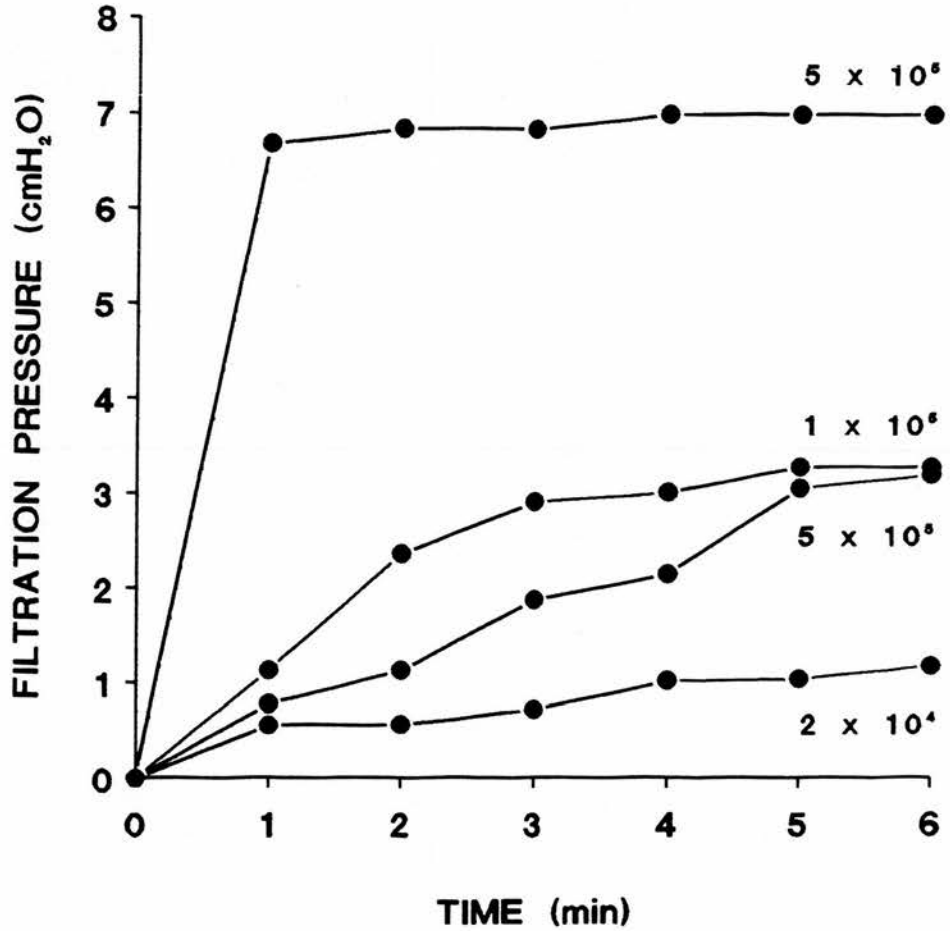


FIGURE 2.6
The effect of neutrophil concentration on filtration pressures developed over 6 minutes filtration in the constant flow filtration system. Mean values for 3 experiments are shown.

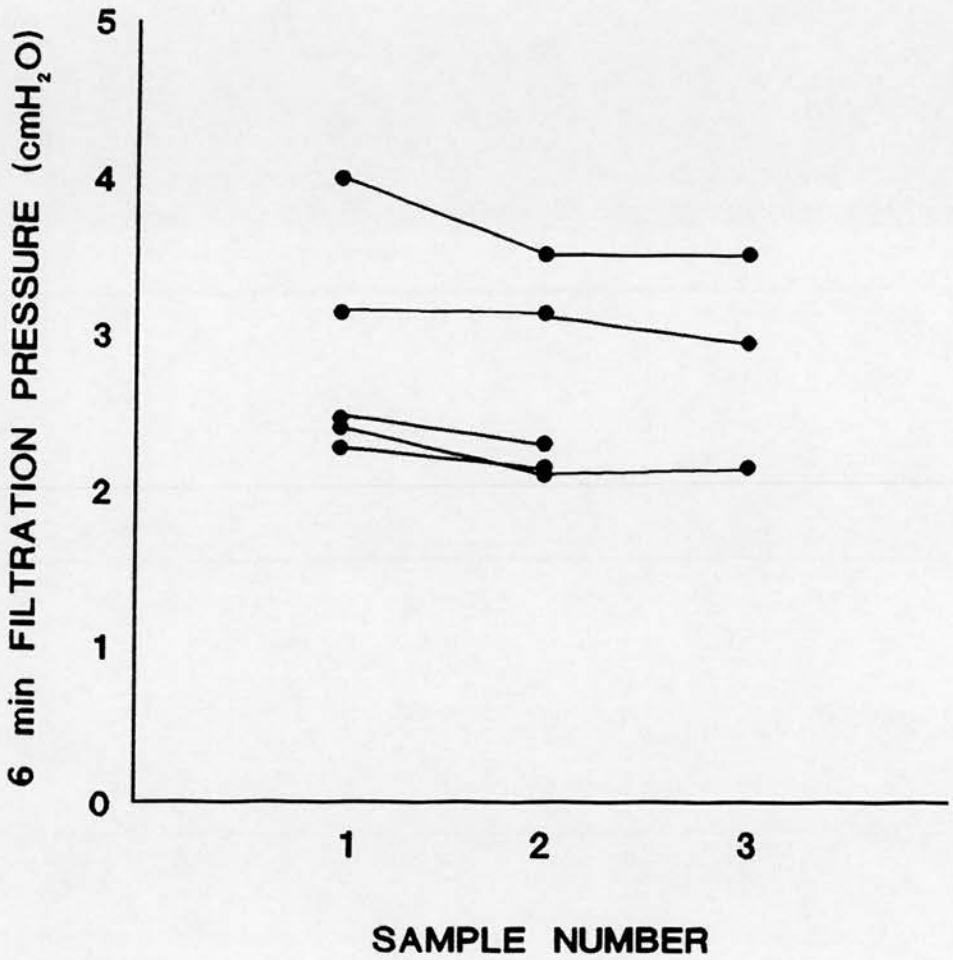


FIGURE 2.7
Figure demonstrating the reproducibility of neutrophil filtration at a concentration of $1 \times 10^5.ml^{-1}$. Aliquots of neutrophil suspensions were filtered at 15 minute intervals on 5 occasions. The data is expressed as the pressure developed after 6 minutes filtration.

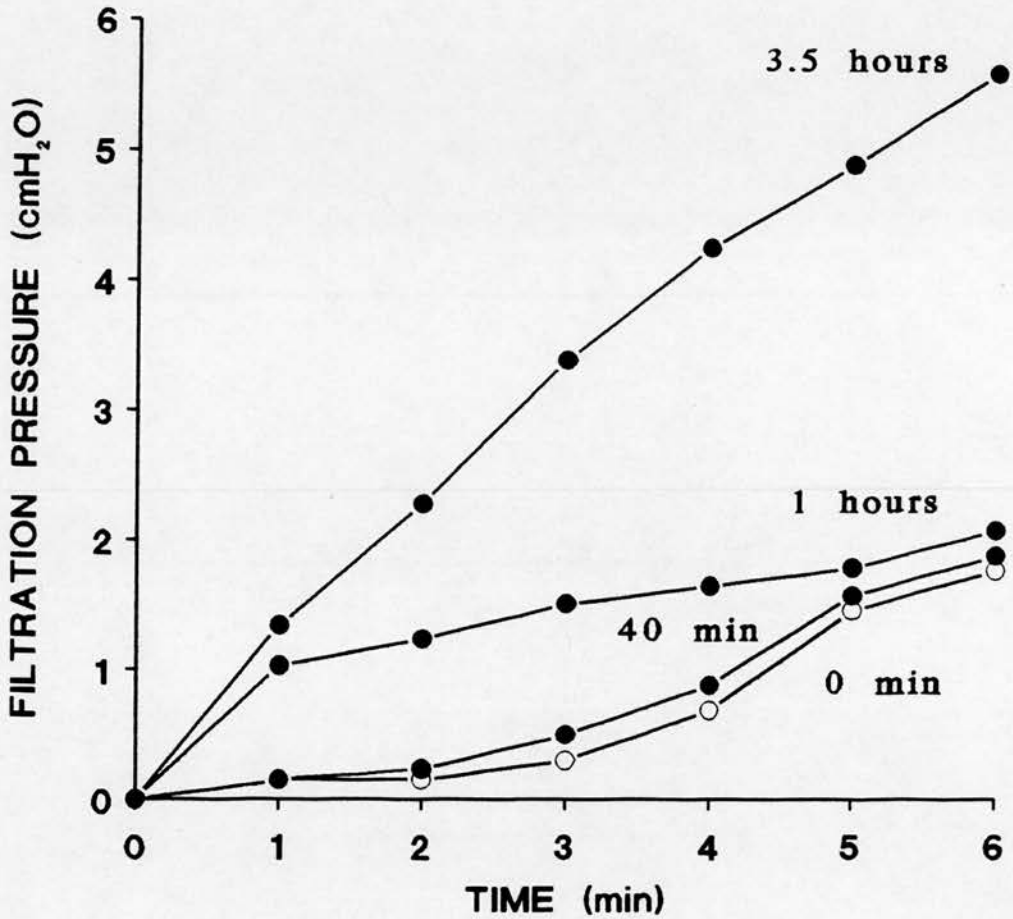
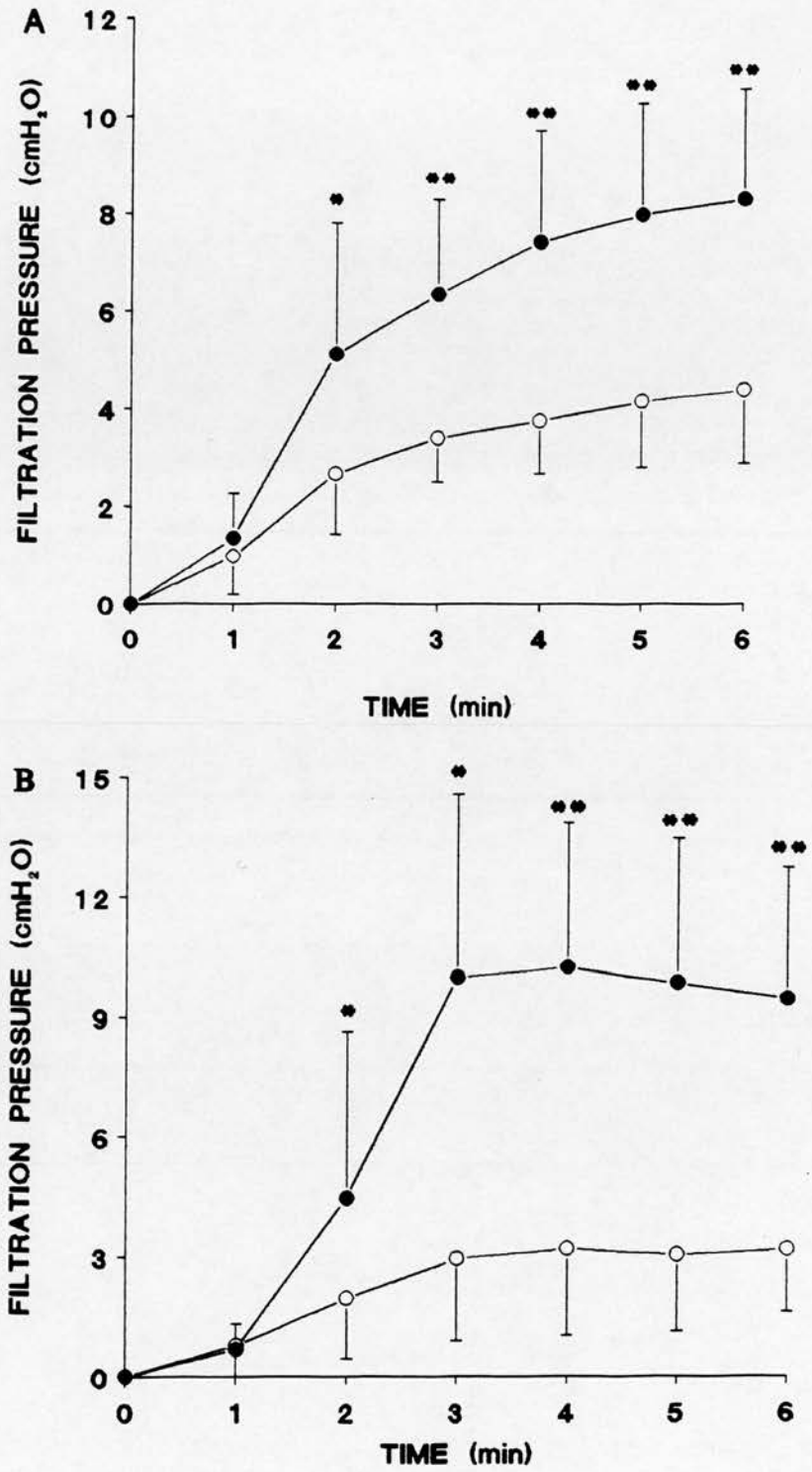


FIGURE 2.8
Mean filtration pressures developed by neutrophils demonstrating the effect of increasing time on neutrophil deformability. Neutrophils deformability was assessed immediately and at 40 minutes, 1 hour and 3.5 hours following isolation from blood on 3 separate occasions.



FIGURES 2.9a and b

The effect of stimulation with (a) 5 $\mu g.ml^{-1}$ PMA (n=7) or (b) 10^{-7} M fMLP (n=8) on the filterability of neutrophils. Stimulated neutrophil (●) developed higher pressures than control cells (O). Mean values with bars representing 1 standard deviation are shown. Compared to control, *$p<0.05$, ** $p<0.01$.

TABLE 2.1

ASSESSMENT OF HARVESTING REAGENTS FOR THE PRESENCE OF BACTERIAL LIPOPOLYSACCHARIDE (ENDOTOXIN) USING THE LIMULUS LYSATE ASSAY

	TEST RESULT		
REAGENT	run 1	run 2	run 3
Percoll (Pharmacia)	-	+	-
6% dextran-70 (pharmacy)	-	-	-
1.8% saline (pharmacy)	-	-	-
sterile PBS (pharmacy)	-	+	-
PBS/0.5% BSA (in house)	-	-	-
distilled water (in house)	-	-	-

- $<0.1 ng.ml^{-1}$, + $>0.1 ng.ml^{-1}$ endotoxin.

TABLE 2.2

THE SPONTANEOUS AND STIMULATED OXYGEN RADICAL PRODUCTION BY NEUTROPHILS

	SPONTANEOUS	PMA $0.1\ \mu g.ml^{-1}$	PMA $1\ \mu g.ml^{-1}$
H_2O_2 (nM $2.5x10^5$ PMN.2hrs)	4.05 (3.2)	20.43 (9.5)***	21.44 (12.8)***
O_2^- (nM $2.5x10^5$.PMN.2hrs)	2.59 (2.7)	15.82 (16.0)***	15.23 (13.5)***

MEAN (SD), n=9.
comparing stimulated with control *** $p<0.001$

TABLE 2.3

DOSE EFFECT OF *IN VITRO* SMOKE EXPOSURE DEMONSTRATED BY MEASURING CARBOXYHAEMOGLOBIN (COHb) LEVELS IN EXPOSED WHOLE BLOOD SAMPLES

NUMBER OF PUFFS	REPLICATES	
SHAM EXPOSED	2.0	2.1
3	7.7	7.9
5	11.7	11.8
7	16.8	16.5

TABLE 2.4

REPRODUCIBILITY OF *IN VITRO* SMOKE EXPOSURE MEASURED AS CARBOXYHAEMOGLOBIN (%COHb) LEVELS IN EXPOSED WHOLE BLOOD SAMPLES

SHAM EXPOSED	SMOKE DOSE	SMOKE EXPOSED		
(%COHb)	(puffs)	(%COHb)		
1.4	2	3.8	3.9	3.9
0.2	4	6.2	6.6	6.2

TABLE 2.5

BUFFER AND WHOLE BLOOD pH VALUES FOLLOWING *IN VITRO* SMOKE EXPOSURE

BUFFER			WHOLE BLOOD		
	SHAM	SMOKE		SHAM	SMOKE
CONTROL	EXPOSED	EXPOSED	CONTROL	EXPOSED	EXPOSED
7.35	7.25	7.07	7.12	7.52	7.53
-	7.13	6.98	7.14	7.52	7.57
			7.16	7.48	7.54

2.5 DISCUSSION

2.5.1 NEUTROPHIL HARVESTING

The neutrophil harvesting procedure used in this thesis involved dextran-aided sedimentation of erythrocytes, plasma/Percoll density gradient separation, and hypotonic lysis to remove contaminating erythrocytes. This technique was adopted to allow isolation of neutrophils under stringent sterile conditions for reinjection into human volunteers for *in vivo* kinetic studies. This would not have been possible with the CCE or flow cytometric techniques. The plasma/Percoll harvesting technique was adopted preferential to the use of Ficoll-Hypaque density gradients. Moreover, all reagents utilised for neutrophil isolation in this study were tested to be as endotoxin free (Table 2.1). Hypotonic lysis of erythrocytes was necessary as *in vitro* labelling with ^{111}Indium-oxine would label any erythrocytes present.

The isolation procedure provided neutrophil populations of high purity and viability. The *in vitro* function of the harvested neutrophils was preserved as assessment of oxygen radical production and protease release revealed low spontaneous functional activity (Table 2.2 and section 2.4.2). This was also confirmed by morphologic examination under light and electron microscopy with the majority of cells maintaining a normal spherical appearance (Figure 2.5). Moreover, MacNee (1989d) and Selby (1991a) and colleagues observed labelled neutrophils harvested using the same isolation technique as in this thesis behaved the same as native unlabelled neutrophils in the sense that low pulmonary sequestration was observed which was not correlated with the subsequent liver and spleen neutrophil sequestration which could have indicated cell activation (Selby, 1991a). Furthermore, neutrophils harvested in this way could be induced to undergo shape change (not shown) and to release their complement of anti-bactericidal agents by addition of the stimulant PMA (Tables 2.2 and section 2.4.2)

2.5.2 NEUTROPHIL FILTRATION

The constant flow filtration system showed good reproducibility (Figure 2.7). A cell concentration of 1 x $10^5 ml^{-1}$ was selected for further studies as it allowed measurement of both the filtration pressures developed by passive and stimulated neutrophils on the same scale of the recorder (Figures 2.9a and b).

As the present studies were investigating the hypothesis that smoke exposed neutrophils have a reduced ability to deform, obtaining a full pressure/time curve of cell filtration, consisting of all three phases described by theoretical analysis (Chien,

1983; Nash, 1990; Reinhart, 1987; Schmalzer, 1983), was important. The final section of the curve is believed to be dominated, with increasing filtration time, by the least deformable portion of cells (Chien, 1983; Nash, 1990). Hence for all filtrations the pressures were monitored and analysed over 6 minutes.

Stimulation of neutrophils, either with the protein kinase C activator PMA or the synthetic chemotactic peptide fMLP, resulted in an increase in the filtration pressures (Figures 2.9a and b respectively). A bimodal response occurred following treatment with fMLP stimulation with an initial rapid increase in filtration pressures, which peaked within 1-3 minutes of addition of the stimulant, and was followed by a gradual decline (Figure 2.9b). By measuring the rate of the pressure increase (P_{grad}) from the gradient of the initial pressure rise, the fMLP-induced increase in filtration pressures (14.9 cm $H_2O.min^{-1}$) occurred more rapidly than following PMA stimulation (5.4 cm $H_2O.min^{-1}$). This reflected a more marked reduction in neutrophil deformability with fMLP stimulation than following the addition of PMA. Neutrophil deformability did not improve following PMA stimulation within the 6 minutes of measurement (Figure 2.9a). The bimodal pattern which occurred with fMLP stimulation has been reported by others (Frank, 1990a), and reflects actin polymerisation and depolymerisation (Belloc, 1990; Frank, 1990a; Howard, 1984; Howard, 1985; Wallace, 1984). The reduced cell deformability observed with both PMA and fMLP stimulated neutrophils is likely to reflect a change in cell shape consequent to a cytoskeletal change and the formation of pseudopods (Bochsler, 1992; Howard, 1985; Watts, 1991). Shape change produces unfolding of the membrane ruffles which reduces the excess surface area available for cell deformation. Also an increase in the maximum diameter would hinder cell transit through pores of smaller calibre. Thus cell shape is an important determinant of cell filterability.

Neutrophils in a blood sample have already spent part of their allotted life span (7 hrs) in the circulation (Wintrobe, 1981). The time required to isolate pure cell populations adds more time. Therefore, the cells whose function is assessed *in vitro*, or used in kinetic studies *in vivo*, represent an older population. An effect of time was evident from a decline in neutrophil deformability when measured over a 3.5 hour period (Figure 2.8). The increase in filtration pressures recorded may have resulted from spontaneous activation of the cells during the course of the experiment as reported by others (Frank, 1990b; Nash, 1988a) as viability was maintained. Using a Cell Transit Analyser to measure the transit times of individual neutrophils,

Frank and Tsai (1990) found only 5% of the cells measured a slower transit time when studied immediately. By 5 hours, however, 30% had extended transit times. Although the transit time for the majority of the cells was unchanged, this subpopulation of stiffer cells would, during the filtration of a cell suspension using a constant flow filtration system, come to dominate the filtration curve, and thus reduce the apparent filterability of the whole cell suspension (Chien, 1983; Nash, 1990). Frank and Tsai (1990) were able to minimise the effect of time by recording the median, rather than the mean, cell transit time thereby reflecting the deformability of the major cell subpopulation.
For the present studies the effect of time was taken into consideration by filtering aliquots of harvested neutrophils in a random order or, if the pressure was expected to be increased following treatment such as stimulation or smoke exposure, the treated samples were filtered before the control samples.

The effect of temperature on the measurement of neutrophil deformability was not investigated in the present study. However, the filterability of neutrophil suspensions at 23° and 37°C was compared by Downey and Worthen (1988) using a similar constant flow filtration system. They observed a higher retention of neutrophils in micropore filters at 37°C than 23°C, which could be explained by a decreased viscosity of the suspension at 37°C resulting in a decreased perfusion pressure. Hence when neutrophil retention in the filter was related to perfusion pressure, the retention was the same. Aoshiba and colleagues (1993) compared neutrophil filterability across a wider range of temperatures (4°C to 40°C) and observed a significant decrease in cell filterability with decreasing temperature, but a relatively small difference between 23°C and 37°C. By disruption of the actin filaments using cytochalasin B, the authors were able to demonstate the decreased filterability during cold exposure was due to alterations in intracellular actin filaments. In agreement with these studies, Evans and Yeung (1989) reported, from micropipette aspiration experiments, a minimal increase in neutrophil viscosity when temperatures were lowered from 37°C to 23°C, although a marked increase in cell viscosity was observed when temperatures were lowered from 23°C to 10°C. Chien (1984) found the elastic behaviour of the cell was not altered by a change in temperature (40-10°C). Moreover, as the filtrations in this study were all performed at an ambient temperature (21-23°C), the pressures developed were directly comparable.

2.5.3 *IN VITRO* SMOKE EXPOSURE

Only the gas phase of cigarette smoke was used for the work in this thesis. Although particulate matter in cigarette smoke is known to be deposited in the lungs (Dalhamn, 1968), a direct effect of smoke particles on cells within the pulmonary microvasculature would not be expected.

As discussed above, some of the volatile components of cigarette smoke, particularly the water soluble components, are likely to be absorbed in the mouth or in the upper airways, and thus prevented from reaching the deeper parts of the lungs. The smoke exposure system used in these studies does not account for such loss. Moreover, no attempt was made to mimic the presence of an epithelial/endothelial barrier. However, to investigate the effect of cigarette smoke on neutrophil deformability it was not deemed possible, or of primary importance, to attain near physiological conditions. However it was considered of relative importance to expose cells directly to fresh smoke considering the acute effect of *in vivo* smoking on neutrophil pulmonary transit (MacNee, 1989d).

Cigarette smoke condensate is frequently used for exposure of isolated cells in culture or suspension to cigarette smoke. However, cigarette smoke dissolved in a solvent is not a good physiological comparison for *in vivo* exposure. The rocker system divised by Voisin and colleagues (1977) for cells in culture is not suitable for cells free in suspension. Hence the tonometer system was developed to allow exposure of cells in suspension directly to cigarette smoke. As the suspension media of the cells would, to some extent, absorb the water soluble components of vapour phase cigarette smoke (Church, 1985; Tsuchiya, 1992), the cells would be bathed in some aqueous smoke extract as well as exposed to fresh smoke.

To monitor the amount of smoke delivered to cell suspensions in the tonometer, the measurement of COHb levels in similarly exposed whole blood samples was chosen. A specific measure of smoke exposure performed on the cell suspension itself may have been preferential. However, although COHb levels in blood are not specific to cigarette smoke, as carbon monoxide is generated by the combustion of petrol or coal for example which would also increase blood COHb levels, it is a simple measurement which can be performed routinely and at reasonable cost. Furthermore, the use of blood COHb levels had the advantage of comparing the exposure dose delivered *in vitro* to the levels attained by acute and chronic smoking *in vivo*. The contribution of enviromental carbon monoxide to the raised COHb in whole blood generated in the exposure system employed would be negligible. The carbon

monoxide in smoke before and after passage through a Cambridge filter (to remove particulate matter) was reported to be virtually the same (Dalhamn, 1968).
The COHb levels measured in samples of blood exposed to a constant dose of vapour phase cigarette smoke (Table 2.4) demonstrates the reproducibility of the system. The maximum exposure level chosen (5 puffs) for subsequent studies produced similar COHb levels as those reported in heavy smokers (Russell, 1973).

The pH values measured in buffer or whole blood altered following exposure to air, and were further altered following smoke exposure in the tonometer for 4 minutes (Table 2.5). However, the levels were still within the physiological range and within the range found to have no affect on neutrophil function (Aoshiba, 1993; Chien, 1984).

In summary, the studies in this chapter demonstrate the ability to obtain a pure, viable population of quiescent neutrophils using the plasma/Percoll harvesting technique; reliably measure the deformability of such populations using a constant flow filtration system; and reproducibly expose cell suspensions to the vapour phase of cigarette smoke *in vitro*.

CHAPTER 3
A COMPARISON OF NEUTROPHIL FILTRATION *IN VITRO* AND *IN VIVO* IN THE LUNGS IN MAN

3.1 INTRODUCTION

In man, isolated leucocytes may be radiolabelled, reinjected into the patient, and their localisation at sites of inflammation imaged externally for diagnostic purposes. Initially used to localise intra-abdominal abscesses (Coleman, 1980), these white cell scanning techniques have subsequently been used to assess the presence of inflammation in the bowel and also the lung parenchyma (Fowler, 1983; Saverymuttu, 1983b). Moreover, unlike the intra-abdominal abscess, both the bowel and lung, when inflamed, allow granulocyte egress. Migrated neutrophils can then be detected by the radioactivity in faeces and sputum respectively.

The acquisition of the scintillation image is frequently performed several hours after reinjection of radiolabelled cells to allow the cells to equilibrate in the circulation and migrate to sites of injury or infection. However, these techniques have recently been extended to allow real-time imaging or counting of the passage of radiolabelled blood cells through the lungs (MacNee, 1989d; Martin, 1982; Muir, 1984; Selby, 1991a & 1991b). An essential feature of such studies is measurement of the very first passage of cells through the circulation and the lungs.

By comparing the measurement of both the 'filtration' of radiolabelled neutrophils *in vivo* through the lungs, and simultaneously the measurement of the deformability of cells from the same aliquot *in vitro*, it may be possible to demonstrate the influence of cell deformability on neutrophil transit through the pulmonary microvasculature.

3.2 AIMS

The purpose of this study was to determine, firstly whether the physiological sequestration of neutrophils in the pulmonary circulation is influenced by their ability to deform, and secondly whether a change in neutrophil sequestration, such as observed in pathological conditions, is reflected by a corresponding change in neutrophil deformability.

3.3 METHODS

3.3.1 NEUTROPHIL HARVESTING

Neutrophils were extracted from 60 ml of ACD anticoagulated whole blood, as described in detail in section 2.3.1. The harvesting procedure was carried out under sterile conditions in a laminar-flow cabinet (Howorth Air Engineering LTD, Farnworth, UK) using reagents and containers which were either shown to be endotoxin free by the manufacturers, or tested using the Limulus Amoebocyte lysate assay (E-Toxicate, Sigma) to contain undetectable levels of endotoxin (<0.005 $ng.ml^{-1}$). Isolated neutrophils were suspended in 5 mls PBS for labelling with indium-111 (^{111}In)-oxine (Thakur, 1977). An aliquot of the cell suspension (1 x 10^6 cells) was taken prior to labelling to measure neutrophil deformability (see below).

3.3.2 NEUTROPHIL RADIOLABELLING

Commercially prepared ^{111}In-oxine (Amersham International PLC, Amersham UK)(18-30 megabequerels, MBq) was used to label the remaining aliquot of cells by drop-wise addition to the cell suspension. Following a 10 minute incubation period to allow cell uptake of the isotope, the cell suspension was centrifuged, the supernatant decanted but retained, and the cell pellet resuspended in 1 ml of autologous plasma (Thakur, 1977). The ^{111}In counts in the cell pellet and the supernatant, representing cell associated, and non-cell associated ^{111}In respectively, were measured in a Compugama CS gamma-well counter (model 1282, Universal Gamma Counter, Turku, Finland) at an emission peak of 247 KeV, and analysed using an Olivetti PCS 286 personal computer and Ultroterm-2 Terminal Emulator software for analysis. From the indium counts the percentage labelling efficiency could be calculated as:

$$\frac{\text{COUNTS IN SUPERNATANT}}{\text{COUNTS IN CELL SUSPENSION + SUPERNATANT}} \times 100$$

3.3.3 ERYTHROCYTE RADIOLABELLING

Erythrocytes were labelled by a combination of an *in vitro* and *in vivo* labelling technique (Smith, 1975). Technitium as pertechnate moves freely in and out of the erythrocytes. Reduced technitium, however, does not readily cross the cell membrane and binds irreversibly with haemoglobin or other components within the erythrocyte. The use of stannous ions to reduce the pertechnate was thus employed for the

purpose of erythrocyte radiolabelling with technetium-99m (^{99m}Tc)-pertechnate. A commercial stannous pyrophosphate kit (stannous chloride/sodium pyrophosphate, Mallinckrodt Diagnostica BV, Petten, Holland) containing 2 mg stannous ion was reconstituted with 10 mls of sterile saline (0.9%), and a 2 ml aliquot was injected intravenously in each subject. Twenty minutes thereafter a 4 ml sample of venous blood was withdrawn into a sterile syringe containing 75-110 MBq ^{99m}Tc-pertechnate and 100 units of heparin (1.5 ml)(Radiopharmacy, Royal Infirmary, Edinburgh). The syringe was mixed for 10 minutes to allow uptake of the radionuclide by the erythrocytes. Prior to re-injection labelled erythrocytes were mixed with the ^{111}In-labelled neutrophils for reinjection.

3.3.4 MEASUREMENT OF NEUTROPHIL LUNG KINETICS

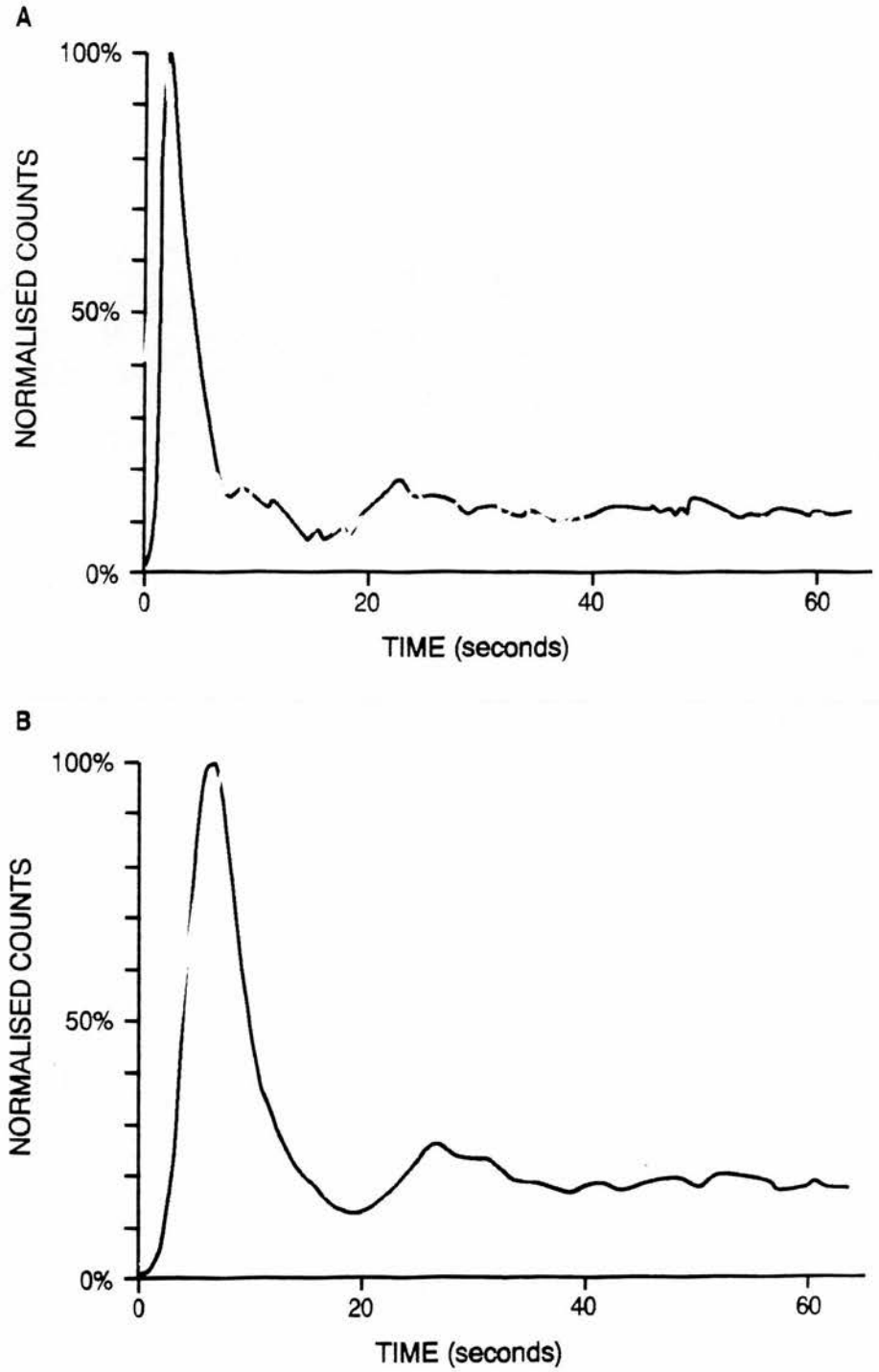
IMAGING OF NEUTROPHIL PULMONARY TRANSIT

The subjects (see below) were positioned semi-supine with a low energy collimator of a large field of view gamma camera (Siemens-Gammasonics, Erlanger, Germany) in an anterior projection (MacNee, 1989d). The gamma camera was on-line to a LINK 1253 MAPS 2000 computer (LINK System, High Wycombe, UK) for data collection and analysis. The radiolabelled erythrocytes and neutrophils were injected into the dead space of an intravenous line sited in the right basilic vein of the subject. The cells were injected into the dead space of the venous line and flushed into the circulation as a bolus with sterile 0.9% saline.

Data were acquired as a dual isotope dynamic study with the gamma camera energy windows at 140 ± 20 KeV and 247 ± 20 KeV for ^{99m}Tc and ^{111}In respectively. The first passage of the radiolabelled bolus of cells through the lungs was recorded as 60 x 500 millisecond time-frames. Without repositioning the subject, image acquisition was continued for a further 29 minutes at 60 second time frames.

3.3.5 ANALYSIS OF *IN VIVO* NEUTROPHIL KINETICS

The neutrophil pulmonary kinetics were, for the majority of studies, analysed by Dr C Selby. Using the images acquired, regions of interest (ROI) were drawn with a light pen around the right ventricle (RV) and the left lung, from which time/activity curves were obtained for the first passage of cells (Figures 3.1a and b) and for the remaining 29 minutes of acquisition (MacNee, 1989c).



FIGURES 3.1a and b.
Time/activity curves of ^{99m}Tc-labelled erythrocytes (solid lines) and ^{111}In-labelled neutrophils (dotted lines) on their first passage through (a) the right ventricle and (b) the left lung in man. The data is obtained immediately following reinjection and is normalised to the peak count for each curve.

The transit time of ^{99m}Tc-erythrocytes from the RV to the left lung were determined from these ^{99m}Tc time/activity curves. A gamma variate function was fitted to the curves for the RV and left lung, which excluded re-circulating cell activity. The mathematical first moment of each curve was obtained, and the erythrocyte transit time determined as the difference between the first moment of the RV and left lung curves (MacNee, 1989c; Muir, 1984).

The first pass of ^{111}In-neutrophils through the ROI was assessed relative to the passage of ^{99m}Tc-erythrocytes. From the time-activity curves from the RV, the ratio of ^{111}In to ^{99m}Tc counts was determined at peak counts. The fitted lung time/activity curve obtained for ^{99m}Tc-erythrocytes passage was then multiplied by this ratio to obtain a calculated curve representing 'zero sequestration' curve for neutrophils (Figure 3.2), which is equivalent to the curve which would pertain if neutrophils passed through the lung like erythrocytes. By integration of this curve, a curve representing a 100% neutrophil sequestration was produced (Figure 3.2). The actual ^{111}In-neutrophil curve observed was normalised to this integral to allow expression of the initial ^{111}In-neutrophil sequestration relative to the ^{99m}Tc-erythrocytes (Figure 3.2). However, as the initial delivery of neutrophils to the lungs is unknown (as assigning a region of interest solely to the RV is not possible), sequestration of neutrophils can not be expressed in absolute terms. Thus initial neutrophil transit was expressed as a comparative rather than an absolute measure of erythrocyte transit (initial normalised sequestration, INS), i.e. as a ratio with 100% neutrophil sequestration given the value of one (Selby, 1991b).

As ^{111}In-neutrophils are only temporarily retained in the lungs, a time-activity curve was acquired for the subsequent 29 minutes. This lung activity curve reflects recirculation, sequestration and (possibly) emigration of neutrophils and can be fitted to the mono-exponential equation:-

$$y = A + Be^{-Ct}$$

where C is the rate constant for the washout. By defining the neutrophil sequestration during the first minute as 100%, a percentage sequestration could be determined from this curve at 10 minutes after reinjection (Muir, 1984) to compare with data obtained at 10 minutes from animal studies (Doerschuk, 1987, 988a &1990a; Hogg, 1988; Martin, 1987 & 1982; Ohgami, 1989; Thommasen, 1984).

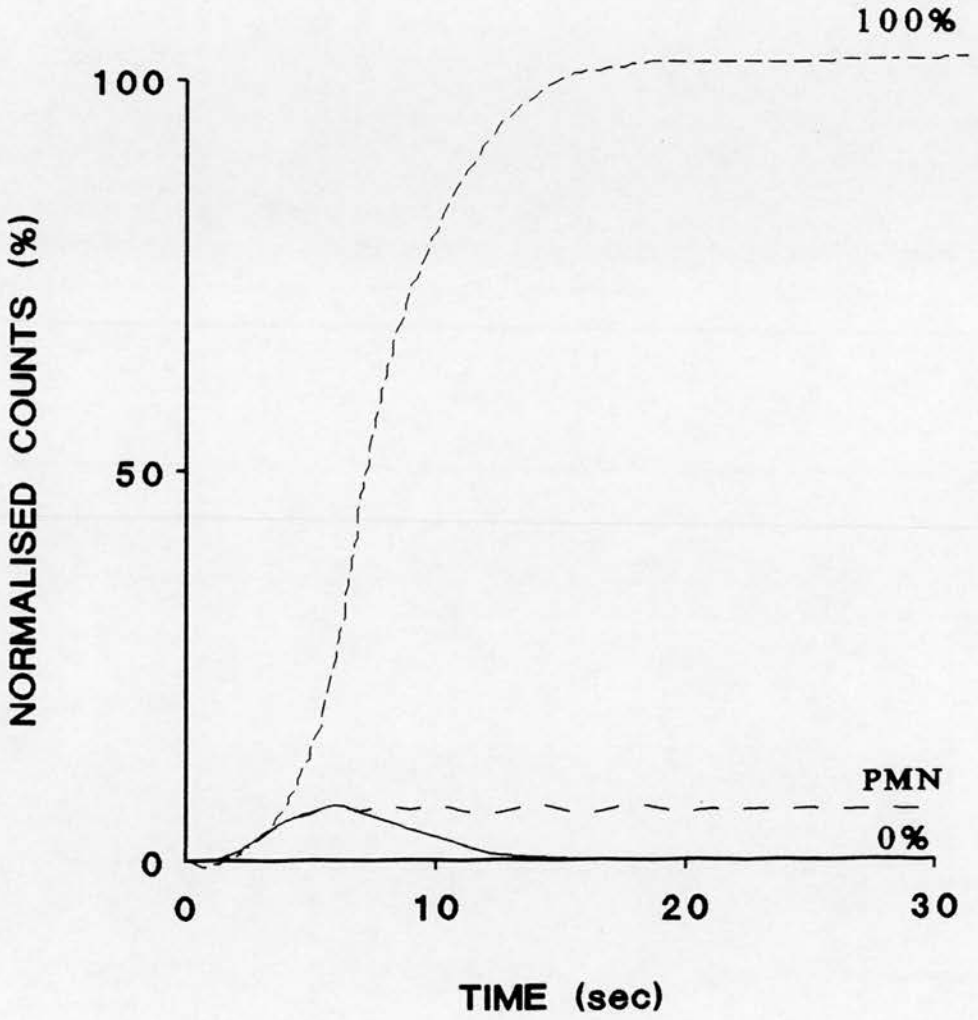


FIGURE 3.2
Curves demonstrating the calculation for the first pass retention of neutrophil passage through the lungs relative to that of erythrocytes following a bolus reinjection. The bottom curve represents the transit of neutrophils through the left lung if they had passed through as erythrocytes do. This calculated curve was obtained by multiplying the erythrocyte lung curve by a ^{111}in-neutrophil: ^{99m}Tc-erythrocytes ratio at peak counts in the right ventricle. Integration of this zero percent retention curve gives a calculated curve representing a nominal 100 percent retention of the neutrophils in the lung. By plotting the actual, observed neutrophil counts normalised to the 100 percent retention curve onto the same graph, the initial passage of neutrophils through the lungs can be obtained comparative to that of erythrocytes and thus expressed as a ratio (Initial Normalised Sequestration ratio (INS), with 100% sequestration given the value of one).

This 10 minute sequestration is a more complex signal as it reflects the circulating, sequestered and potentially any migrating cells.
Blood samples taken 30 minutes after the injection of radiolabelled neutrophils and erythrocytes showed that less than 1% of the infused radioactivity could be detected in the plasma, indicating continued cell association of the radiolabels.

SUBJECTS
Simultaneous measurement of neutrophil *in vitro* filterability, and *in vivo* lung kinetics was obtained in 18 elderly subjects. All subjects gave informed consent for the study which had been approved by the local medical ethical committee, and the Administration of Radioactive Substances Advisory Committee at the Department of Health.

Ten subjects (6 males: aged 68-88 years) without clinical symptoms of pulmonary disease were studied. They had been referred to hospital with a diagnosis of osteoarthritis. Three of the subjects were chronic smokers, but abstained for a minimum of 2 hours prior to the study of neutrophil kinetics. A further 8 subjects (6 males: aged 62-85 years) with COPD diagnosed by respiratory physicians, but in a clinically stable condition were studied. Their forced expiratory volume in 1 sec (FEV_1) was 8-57% of predicted.
Neutrophil deformability was also measured in patients (6 males: aged 54-81 years) presenting with an acute exacerbation of COPD (within 12 hours of admission to hospital), with a FEV_1 18-50.4% of predicted (when stable). Neutrophil deformability was reassessed in these patients prior to discharge when clinically stable.

3.3.7 *IN VITRO* NEUTROPHIL FILTRATION

Neutrophil filterability was assessed using a positive pressure filtration system, described previously (section 2.3.3) as a measure of the deformability of a population of neutrophils. Each aliquot of harvested neutrophils was diluted to a concentration of 1×10^5 with PBS containing 0.5% bovine serum albumin (PBS/BSA) and filtered, in duplicate immediately following acquisition of *in vivo* neutrophil kinetics. In these studies the filtration pressures developed at each minute were calculated, as a measure of cell deformability, and also the gradient of the initial pressure rise (referred to as P_{grad}).

FILTRATION OF RADIOLABELLED CELLS

Two *in vitro* filtration experiments were performed with radiolabelled cells prepared as for the *in vivo* kinetics experiments:

(1) To determine whether the radiolabelling procedure and the presence of the radionuclide affected neutrophil deformability, the filterability of ^{111}In-labelled neutrophils was compared with an unlabelled aliquot of cells. The experiment was performed in duplicate on occasions.

(2) To examine the kinetics of neutrophil filtration *in vitro* through 5 μm pore membranes, relative to the filtration of erythrocytes. A sample of mixed ^{111}In-labelled neutrophils and ^{99m}Tc-labelled erythrocytes were diluted to 4% with PBS and filtered as described in section 2.3.3. The filtrate effluent was collected into tubes at minute intervals, which were gamma well counted on two channels (10-70 KeV and 80-300 KeV) to determine the amount of ^{99m}Tc and ^{111}In radioactivity respectively. The percentage of the counts for each radionuclide, corresponding to each cell type, was calculated relative to the radioactivity of the infusate. Radioactive counts on the filter were also measured to establish cell retention in the filter after 6 minutes' filtration.

3.3.7 STATISTICAL ANALYSIS

Measurement of association between pairs of variables was by Spearman's rank correlation where the hypothesis of a positive relationship between *in vivo* initial sequestration, and *in vitro* filterability allowed one-tailed significance testing to be performed. Comparison of paired data was done by Wilcoxon's matched-pair signed-ranked testing and that of unpaired data was by the Mann-Whitney U test (Snedecor, 1974).

3.4 RESULTS

3.4.1 NEUTROPHIL HARVESTING AND RADIOLABELLING

The harvesting procedure produced 94 ± 2.6% pure neutrophils (n=28), with less than 2% erythrocyte contamination. Microscopic examination revealed the cells were 98% viable with no evidence of aggregation or shape change. Labelling efficiency of neutrophils with ^{111}In-oxine was 69 ± 9% (n=28).

3.4.2 *IN VITRO* FILTRATION OF RADIOLABELLED CELLS

(1) The filtration of both ^{111}In-labelled and unlabelled neutrophils was reproducible. ^{111}In-neutrophils developed slightly higher filtration pressures than unlabelled cells (Figure 3.3). The initial pressure rise, measured as the gradient (P_{grad}), was not different between labelled and unlabelled cells (Figure 3.3).

(2) The radioactivity of ^{111}In-neutrophils and ^{99m}Tc-erythrocytes in the effluent following filtration was measured at minute intervals, and the ratio of erythrocytes: neutrophils calculated. In the first minute a greater number of erythrocytes than neutrophils was observed to filter through the membrane (Table 3.1). From the second minute of filtration a steady state was attained, with equal cell numbers for both neutrophils and erythrocytes entering and leaving the filter. Knowledge of the ^{111}In-neutrophil specific activity allowed the number of neutrophils filtered in each minute to be determined (Table 3.1). Also the number of neutrophils retained in the filter was calculated to be 8.56×10^3. With 5×10^5 pores per filter (manufacturers specifications), 1.7% of the pores were estimated to be occupied by neutrophils, assuming only one cell per pore.

3.4.3 *IN VITRO* NEUTROPHIL FILTRATION AND *IN VIVO* NEUTROPHIL KINETICS

Table 3.2 shows the respiratory function and blood gas data for the patients with COPD and the normal subjects.

The kinetic parameters of erythrocyte transit time (TT), the initial neutrophils sequestration (INS) relative to that of the erythrocytes and the subsequent neutrophil lung washout data (10 minute sequestration and C) for these subjects are summarised in Table 3.3. These parameters were the same for the two groups.

In the lungs of normal elderly subjects the filtration pressures developed after 6 minutes filtration (P_6) *in vitro* correlated with the INS measured *in vivo* (Figure 3.4a). However, neither the rate of washout (C) nor the percentage sequestration at 10 mins, correlated with the *in vitro* filtration of neutrophils ($r=-0.4$ to $r=0.4$, $p>0.05$).

Neither was there a significant relationship between erythrocyte transit time, or the sequestration at 10 minutes and INS (r=-0.5, p=0.2; r=0.5, p=0.2 respectively).
In contrast to normal subjects, in patients with stable COPD the filtration pressure measured after 6 minutes (P_6) did not correlate with the INS (Figure 3.4b). However, the initial pressure rise (P_{grad}) correlated with INS for COPD patients (Figure 3.5b), and also in normal subjects (Figure 3.5a).

3.4.4 *IN VITRO* FILTRATION OF NEUTROPHILS FROM PATIENTS WITH EXACERBATIONS OF COPD

The clinical details for patients with acute exacerbation of COPD are listed in Table 3.4. The filterability of harvested peripheral blood neutrophils was assessed for these COPD patients when admitted to hospital during an exacerbation (EXB), and prior to discharge when the patients were clinically stable (STB). Both the P_6 and P_{grad} fell consistently when the patients condition returned to clinical stability (P_6 Figure 3.6: mean P_{grad} EXB 5.96 ± 2.07 cm H_2O min^{-1}, STB 3.07 ± 2.9 cm H_2O min^{-1}; n=10, $P<0.05$).

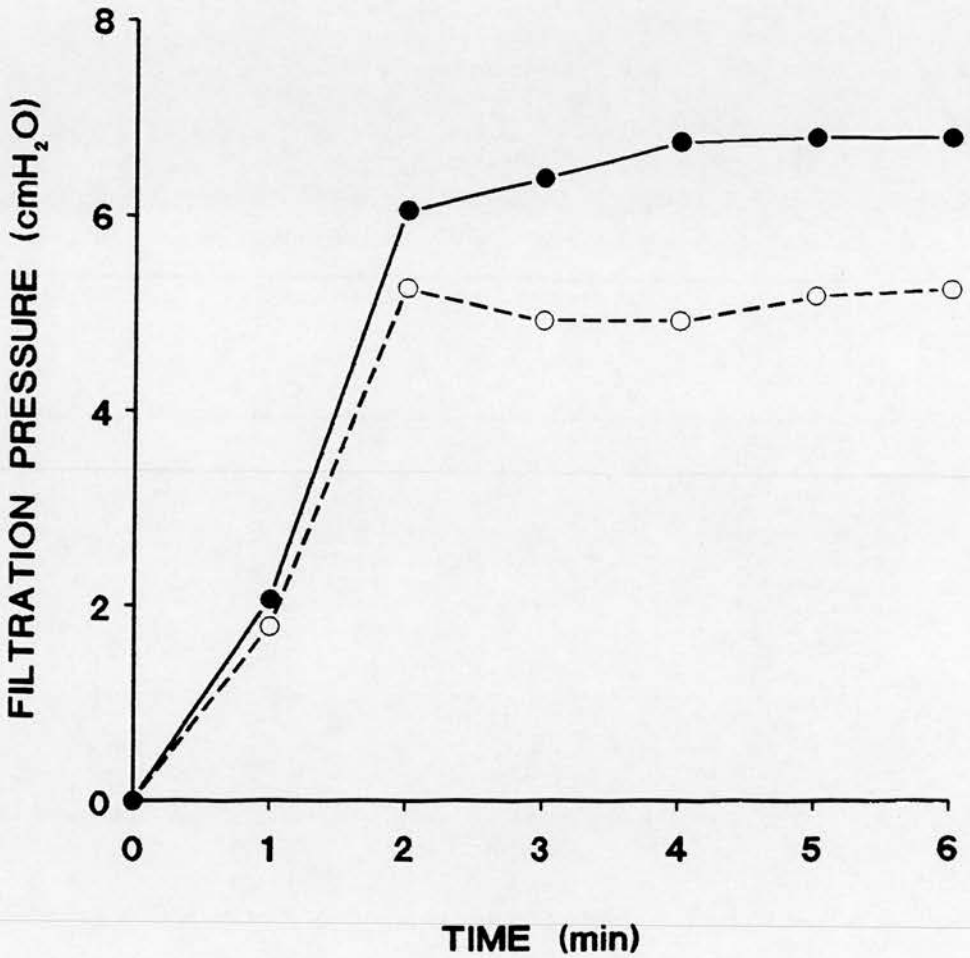
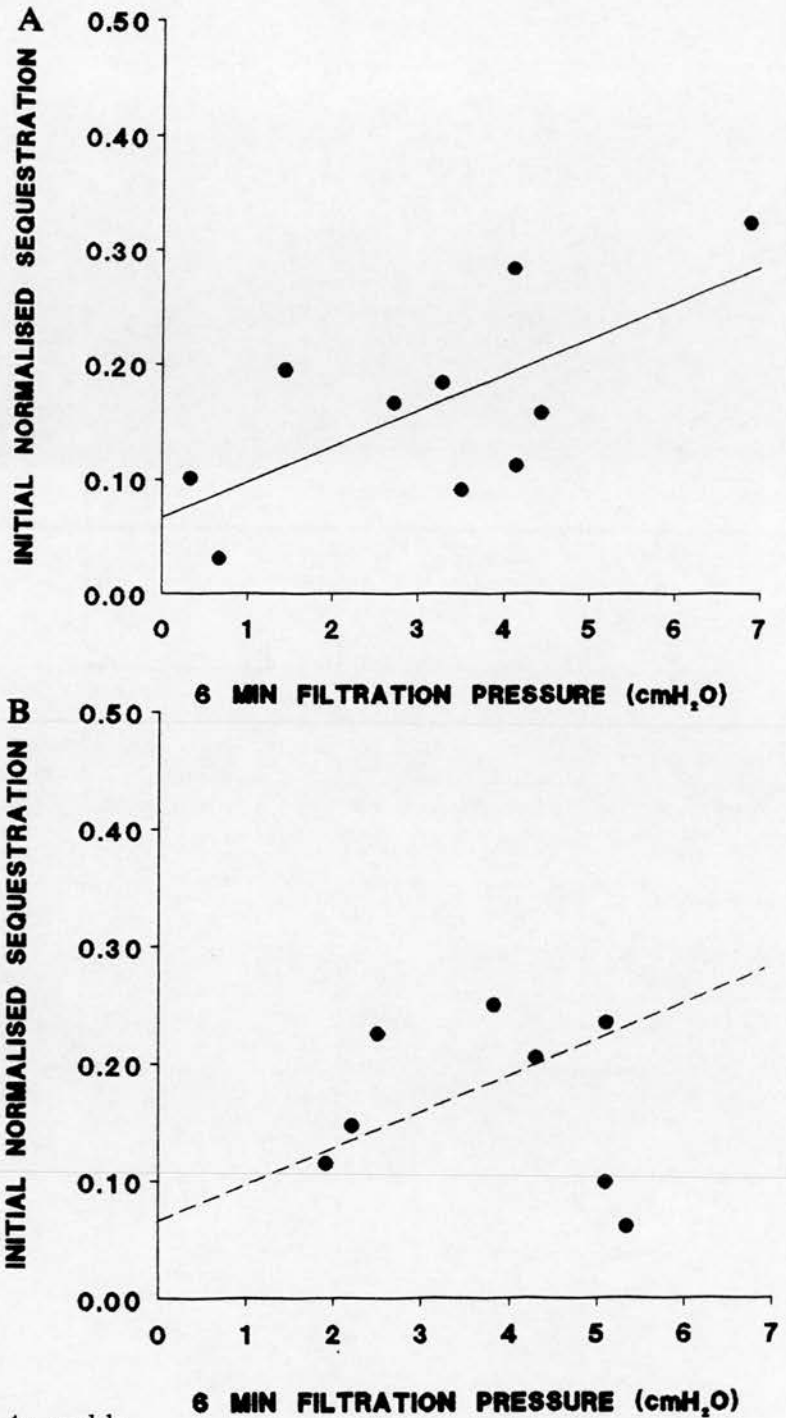
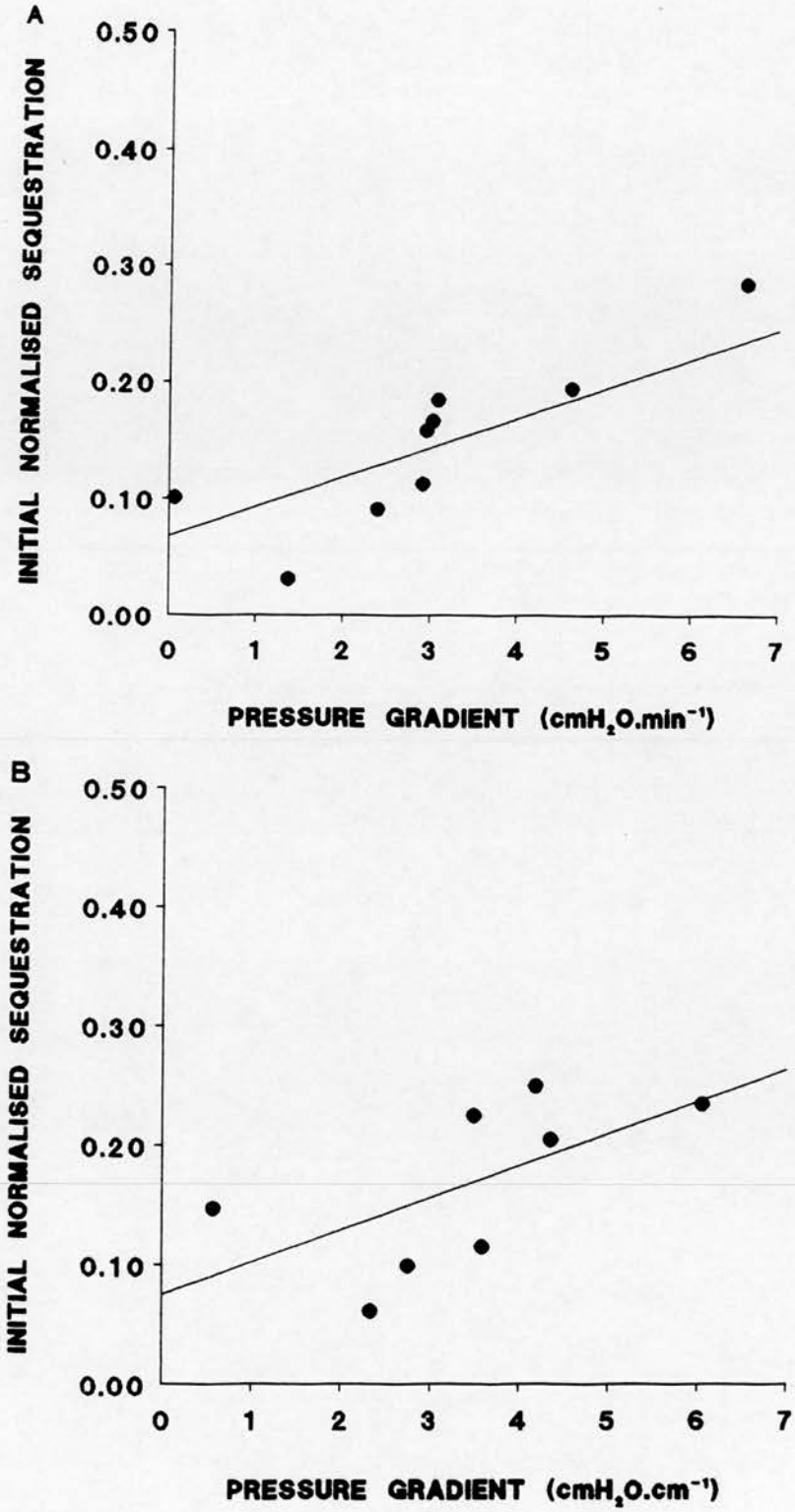


FIGURE 3.3
The pressures developed by ^{111}In-labelled and unlabelled neutrophils over 6 minutes filtration. The results, from two experiments, demonstrate slightly higher filtration pressures for ^{111}In-labelled neutrophils (●) than unlabelled neutrophils (O). The initial pressure rise (P_{grad}) was not different between labelled and unlabelled cells.



FIGURES 3.4a and b

The correlations for (a) 10 normal subjects and (b) 8 patients with stable chronic obstructive pulmonary disease (COPD) between the pressure developed after 6 minutes filtration (P_6) *in vitro* and the simultaneous measurement of first pass lung sequestration (INS) *in vivo*, using cells from the same aliquot. A significant linear relationship between P_6 and INS was established for normal subjects ($r=0.69$, $p<0.02$) which was lost for the COPD patients, although the majority of subjects lie around the regression line for normal patients (as illustrated in Figure 3.4b). The two outlying subjects had the most severe emphysema as determined by computed tomography scanning (Selby, 1991a).



FIGURES 3.5a and b
Correlations between the rate of the initial pressure rise (P_{grad}) measured *in vitro* and the initial first pass sequestration *in vivo* (INS) of neutrophils from the same aliquot in (a) normal subjects and (b) patients in a stable condition of chronic obstructive pulmonary disease (COPD). A significant linear relationship existed for both normal (r=0.9, p=0.001) and stable COPD patients (r= 0.6, p=0.05).

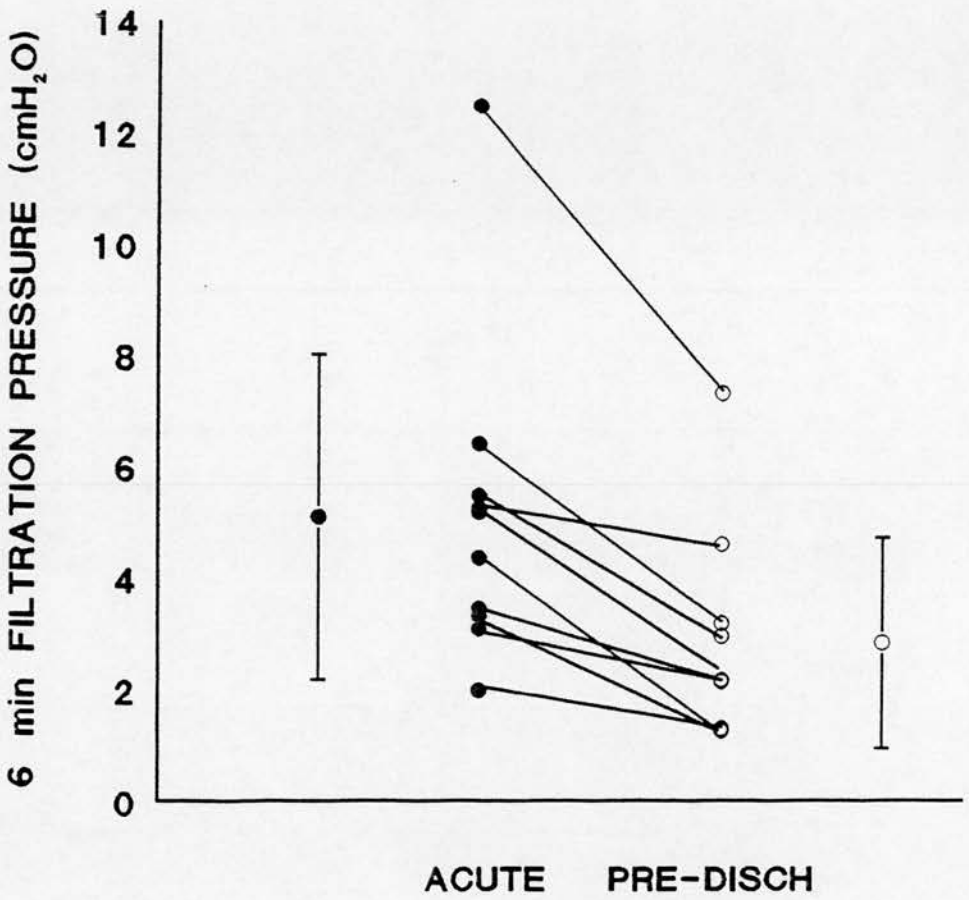


FIGURE 3.6
The 6 minute filtration pressures developed by harvested neutrophils from patients during an acute exacerbation of chronic obstructive pulmonary disease (ACUTE) and repeated when in a stable condition prior to discharge from hospital (PRE-DISCH). A significant improvement in neutrophil deformability was evident as the patients condition improved ($n=10$, $p<0.01$).

TABLE 3.1

FILTRATION OF ^{99M}TC-ERYTHROCYTES AND ^{111}IN-NEUTROPHILS THROUGH A MICROPORE MEMBRANE *IN VITRO*

TIME	FILTRATION PRESSURE	RBC:PMN RATIO	DETERMINED PMN NUMBER [b]
0	0	5.8:1 [a]	
1	20.8	14.8:1	5.48×10^2
2	19.5	9.3:1	1.19×10^3
3	18.4	8.9:1	1.25×10^3
4	17.4	9.9:1	1.18×10^3
5	16.7	9.2:1	1.19×10^3
6	16.8	9.5:1	1.09×10^3
FILTER AT END		1:14.6	8.56×10^3

[a] cell ratio in the suspension prior to filtration.

[b] Neutrophil numbers were determined from the ^{111}Indium activity, knowing the specific activity of the ^{111}In-neutrophil.

TABLE 3.2

CLINICAL DATA FOR NORMAL SUBJECTS AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE [COPD] PATIENTS IN WHOM *IN VIVO* NEUTROPHIL LUNG KINETICS AND *IN VITRO* CELL DEFORMABILITY WAS ASSESSED

	NORMAL SUBJECTS	COPD PATIENTS
n	6M, 4F	6M, 2F
Age [Yr]	77.6 [6.6]	71 [8.9]
FEV_1 [Ltrs]	2.17 [(0.7]	0.76 [0.3] *
FEV_1 of % predicted	91.5 [19.1]	28 [14.8] **
FVC [Ltrs]	3.1 [0.9]	2.3 [0.7]
FVC of % predicted	90.5 [15.6]	60.8 [17.8]
FEV_1 / FVC of % predicted	75.7 [11.6]	32.7 [13.3] *
$P0_2$ [kPa]	11.0 [0.6]	7.7 [1.6] **
Pco_2 [kPa]	4.9 [0.4]	6.1 [0.9] *

MEAN [SD]. FEV_1 is the forced expiratory volume in 1 second. FVC is the forced vital capacity. * $p<0.01$, ** $p<0.001$.

TABLE 3.3

LUNG KINETIC DATA FOR NORMAL SUBJECTS AND PATIENTS WITH STABLE CHRONIC OBSTRUCTIVE PULMONARY DISEASE [COPD]

	NORMAL SUBJECTS	COPD PATIENTS
RBC transit time [s]	4.0 [1.7]	4.2 [0.7]
INS	0.17 [0.09]	0.17 [0.07]
10 min sequestration [%]	66.2 [9.9]	64.2 [10.3]
C [x $10^{-3}.s^{-1}$]	1.77 [0.81]	3.16 [1.76]

MEAN [SD]. INS, initial normalised neutrophil sequestration. C, the rate constant for neutrophil washout from the lungs.

TABLE 3.4

CLINICAL DATA FOR PATIENTS WITH AN ACUTE EXACERBATION CHRONIC OBSTRUCTIVE PULMONARY DISEASE [COPD] AND WHEN STABLE WHERE NEUTROPHIL DEFORMABILITY WAS MEASURED

	ACUTE	STABLE
Age [Yr]	69 [7.0]	
Po_2 [kPa]	6.6 [1.9]	8.1 [2.1] *
Pco_2 [kPa]	7.4 [2.5]	5.9 [1.9] *
$[H^+]$ [nM]	43.2 [6.3]	37.9 [2.9] *
WBC [x $10^9.L^{-1}$]	11.3 [5.1]	9.0 [2.9]
PMN [x $10^9.L^{-1}$]	8.9 [4.8]	5.9 [1.9]
FEV_1 [Litres]		1.0 [0.5]
FVC [Litres]		1.8 [0.7]
FEV_1/VC [%]		56.1 [10.3]

MEAN [SD] * $p<0.05$.
WBC, white blood count. FEV_1, forced expiratory volume in 1 second. VC, vital capacity.

3.5 DISCUSSION

3.5.1 NEUTROPHIL SEQUESTRATION IN THE LUNGS

In 1867 Cohnheim (1867) introduced the concept of neutrophil margination in peripheral vessels in the systemic circulation when he observed leucocytes rolling along vessel walls while erythrocytes remained in the axial stream. Nearly a century later, Athens and co-workers (1961a & 1961b) noted that 15-20 minutes following infusion of di-isopropylfluorophosphate (DFP^{32})-labelled neutrophils, 50% had disappeared from the circulation. The loss was postulated not to be due to cell damage or elution of the radiolabel, but to equilibration in the total blood granulocyte pool, with 50% of the cells present in the marginating pool, and the other 50% remaining in the circulation.

Many studies in animals and in man indicate that the vascular bed of the lungs is an important site of leucocyte sequestration (Bierman, 1951, 1951b, 1952a, & 1952b; Doerschuk, 1987 & 1989; Hogg, 1987 &, 1988; MacNee, 1989b & 1990; Martin, 1987; Muir, 1984; Thommasen, 1984). However, Peters and co-workers (1985a & 1985b) have contested this opinion since they found the lung marginating granulocyte pool in man to be only 10% of the total blood granulocyte pool, compared with 30-50% for the spleen. Moreover, Muir and associates (1984) found a decrease in the lung ^{111}In-labelled leucocytes as a result of exercise, which paralleled the peripheral blood leucocytosis and thus was taken as supportive evidence for the existence of a pulmonary marginating pool of leucocytes. In contrast Peters and co-workers (1992) observed little change in the lung marginating pool when subjects were exercised. However, different techniques were used in these studies to measure leucocyte kinetics. Peters et al (1985b) advice radiolabelling with ^{111}In-tropolonate which can be performed in the presence of plasma, unlike labelling with ^{111}In-oxine, as removing cells from plasma for even a short period of time causes cell activation (Talstad, 1971). However, even with plasma present throughout the harvesting and labelling procedure, these authors found neutrophils were minimally activated resulting in pulmonary sequestration when reinjected *in vivo* (Peters, 1992; Saverymuttu, 1983a). They thus proposed a recovery period was necessary to allow cells to equilibrate in the circulation prior to performing leucocyte kinetic studies. As Muir and colleagues (1984) measured leucocyte lung kinetics only 10 minutes after reinjection of radiolabelled erythrocytes and neutrophils, Peters suggested the release of the neutrophils from the lungs following exercise measured by Muir et al reflected a recovery of cells activated by the harvesting and radiolabelling procedures, and not as a physiological demargination as a result of exercise (Peters, 1992). However, Muir and colleagues (1984) found only a 20-25% sequestration of leucocytes on their

first passage of the pulmonary vasculature, although this is higher than more recent estimates (MacNee, 1989d; Selby, 1991a) possibly reflecting their use of Ficoll-Hypaque density gradients for cell harvesting. Furthermore, the labelled leucocytes responded to exercise in a similar manner as unlabelled cells (Muir, 1984). The methodology chosen by Peters and associates (Peters, 1992) may have been influenced by the background indium levels of circulating ^{111}In-leucocytes, particularly as low levels of indium were used for their studies. Moreover, in their studies the labelled erythrocytes and leucocytes were injected at different times.

3.5.2 NEUTROPHIL PULMONARY KINETICS

The kinetics of neutrophil lung transit has been studied following a bolus reinjection of the cells relative to that of simultaneously reinjected erythrocytes (Hogg, 1988; Markos, 1990; Martin, 1982). Alternatively the time sequence of leucocyte washout from the lungs over minutes or hours has been determined (Haslett, 1987; Worthen., 1987a), or cell influx into the airspace in response to inflammation assessed (Doerschuk, 1990; Gie, 1991; Worthen, 1987a).

Intravital microscopy of the subpleural microcirculation of the dog lung, by Lien and colleagues (1987a), has revealed a wide distribution of neutrophil transit times ranging from 2 seconds to 20 minutes. They observed that fluorescently labelled neutrophils moved through the capillary segments as though from one discrete obstacle to the next rather than in a continuous movement as would occur if they 'squeezed' through the length of each capillary segment (Lien, 1990). Moreover, they found that recirculating cells, i.e. those that entered the field of view 45 seconds after their initial infusion, had a similar distribution of transit times as on their first passage (Lien, 1990). Increasing blood flow by epinephrine infusion, or reducing venous return by inflating a balloon in the caudal vena cava, shifted the distribution of neutrophil transit times towards the faster or slower transit times respectively without altering the median transit times (Lien, 1990). Thus, rather than a progressive shift of all cells into either the faster or slower transit time intervals, depending on the change in blood flow, only a proportion of neutrophils changed from having slow to fast capillary transit times when flow was increased, and vice versa when flow was reduced. The analogy Lien and colleagues (1990) used to explain this phenomenon was that of a barge hitting a sandbank. If the flow or the pressure was high enough, the cells would be pushed over the obstacle. The larger neutrophils or those which are less deformable would be most likely hindered by the 'sandbanks', but could have a rapid transit time once the pressure or flow was

elevated enough. However, the site of neutrophil sequestration was always within the capillaries. By contrast the intravital microscopy of the dog trachea revealed that neutrophil margination in these systemic vessels occurred in post-capillary venules (Lien, 1991).

An unique advantage for studying the kinetics of neutrophils in the lungs is that an intravenous injection of a bolus of cells will arrive at the pulmonary microvasculature in its entirety. Utilising this, it has been possible to study real-time leucocyte and erythrocyte kinetics through the pulmonary microcirculation. Studies using indicator dilution techniques, or external imaging of radiolabelled cells, have shown an increased sequestration of neutrophils in the lungs of animals (Doerschuk, 1987a; Hogg, 1988; Martin, 1987; Ohgami, 1989), and man (MacNee, 1989d; Selby, 1991a) compared with that of erythrocytes. Moreover, these techniques have allowed the influence of local hydrodynamic forces and alveolar pressures to be assessed (Doerschuk, 1988a; Markos, 1990; Martin, 1982).
Martin and associates (1987) found more than 80% of reinjected ^{111}In or ^{51}Cr labelled neutrophils were retained in the lungs of dogs on their first passage, independent of the cardiac output. In human lungs, neutrophil retention on their first passage was approximately 20% (Muir, 1984), with more recent data suggesting a 5-11% first pass neutrophil retention (MacNee, 1989d; Selby, 1991a). This initial sequestration of neutrophils in man, as found for dogs (Martin, 1987d) was unrelated to erythrocyte transit time, and thus independent of blood flow. The washout of neutrophils from the lungs (10 minutes sequestration and C) after their re-injection was, however, affected by blood flow as found previously (Doerschuk, 1987a; MacNee, 1989d; Martin, 1987). The differences in neutrophil sequestration between studies in animals (Martin, 1987) and those in man (MacNee, 1989d; Muir, 1984; Selby, 1991a) may reflect the different techniques used to assess neutrophil kinetics and the fact that the animals were anaesthetised during the acquisition of the scintillation images, whereas the human subjects were awake.

3.5.3 MODELLING NEUTROPHIL PULMONARY KINETICS

The kinetics of leucocyte transit over time has also been modelled *in vitro*, using micropore membranes to mimic the capillary segments. As discussed at length in chapter 1, cell transit through the microvasculature may be influenced by cell deformability, adhesive interactions and/ or hydrodynamic forces. Employing filtration models it is possible to isolate and study the influence of geometric

constraints on the passage of cells which is relevant to the situation in the lungs *in vivo*.

Downey and Worthen (1988) adapted a filtration method established by Usami and colleagues (1975) to investigate the importance of cell size and deformability. In their system pulses of radiolabelled neutrophils and erythrocytes were filtered in buffer through micropore membranes with 5 μm pore diameters (Downey, 1988). Sampling of the filtrate effluent, at pre-determined intervals, allowed the construction of time/activity curves analogous to those obtained by indicator-dilution studies *in vivo* (Markos, 1990; Martin, 1982). Moreover, in the *in vitro* system, the effect of pressure, pore size and temperature could be investigated by measuring the retention of cells in the filter as an end point (Downey, 1988). With this model of the pulmonary microvasculature, Downey and Worthen (1988) measured enhanced filter retention of ^{111}In-neutrophils compared with ^{99m}Tc-erythrocytes which is consistent with *in vivo* studies. The enhanced filter retention of neutrophils compared with erythrocytes was also demonstrated in the present study using radiolabelled cells. The retention of a greater number of ^{111}In-neutrophils (PMN) than ^{99m}Tc-erythrocytes (RBC) in the filter after 6 minutes filtration (PMN 14.6:RBC 1) demonstrates the greater filterability of these cells. That more erythrocytes than neutrophils were present in the effluent in the first minute (PMN 1: 14.8 RBC), compared with the cell ratio in the suspension before filtration (PMN 1: RBC 5.8), confirms this observation. Downey and Worthen (1988) also demonstrated that neutrophil filter retention was dependent on geometric factors such as cell size and deformability, and influenced by haemodynamic factors such as increased perfusion pressure (shear stress) due to either increased flow or erythrocyte hematocrit. Subsequently the same authors also related an increase in the 'stiffness' of neutrophils, measured directly by the 'cell poker' technique, to both the retention of ^{111}In-neutrophils *in vitro* in micropore membranes and *in vivo* in rabbit lungs (Downey, 1990).

3.5.4 *IN VITRO* AND *IN VIVO* NEUTROPHIL 'FILTERABILITY'

In this chapter the importance of cell deformability for neutrophil transit of the pulmonary circulation was investigated by directly comparing the filtration of neutrophils through the pulmonary microvasculature in man to their ability to filter through a micropore membrane *in vitro*. A significant relationship was established between the *in vitro* filterability of neutrophils, as a measure of cell deformability, and the *in vivo* filtration of neutrophils on their first passage through the lung

capillary bed (INS). The INS measured in normal elderly subjects correlated with both the pressures developed after 6 minutes *in vitro* filtration (P_6) and the initial pressure rise referred to here as the gradient pressure (P_{grad}). This highly significant relationship between the initial *in vivo* hold-up of neutrophils and the *in vitro* measurement of cell deformability confirms the importance of cellular deformability for the passage of neutrophils through the pulmonary microvasculature. Moreover, it highlights the relevance of this *in vitro* measurement of cell filtration as a model for neutrophil passage through the pulmonary microvasculature, which occurs for each neutrophil circulation of the body. Additionally, this close relationship implied little involvement of adhesive factors between neutrophils and endothelial cells for INS in normal lungs.

The lack of a relationship between INS and erythrocyte transit time for normal and stable COPD subjects suggests the initial lung sequestration of neutrophils is independent of blood flow. Likewise, the *in vitro* measurement of cell deformability could not be correlated with erythrocyte transit time. In contrast, the later neutrophil sequestration at 10 minutes was found to be directly related to erythrocyte transit time, and therefore inversely related to blood velocity (Hogg, 1988). This was evident from transit times of neutrophils measured in different lung regions. The wide range of regional blood velocities, in the lungs resulted in variations in the regional turnover between sequestered and circulating neutrophils (Hogg, 1988; Martin, 1987). This late sequestration does not, however, reflect the initial sequestration but is comparable to the washout parameters measured in the present study. Hence, it is not surprising that neither INS nor the *in vitro* filtration pressures correlated with 10 min sequestration which is a complex signal comprising several determinants as previously indicated.

The second aim of this chapter was to determine whether a change in INS would be matched by a similar change in cell deformability. To answer this question, measurements of INS and *in vitro* neutrophil filtration were undertaken in patients with stable COPD. Also, in this study *in vitro* neutrophil filtration was assessed for patients with an acute exacerbation of COPD, and compared with a previous study where *in vivo* kinetics (INS) was measured (Selby, 1991a).

Stockley and co-workers (Burnett, 1987; Stockley, 1982) reported enhanced neutrophil proteolytic activity and the presence of immunoreactive elastase in patient's plasma, indicating neutrophil activation in patients with COPD. As the deformability of activated neutrophils is known to be reduced (section 2.4.3)(Frank, 1990a; Nash, 1988a), an increased INS would be predicted.

In contrast to normal subjects, no significant correlation was established between P_6 and INS for patients with stable COPD, although the relationship between the INS and the initial pressure rise (P_{grad}) measured by *in vitro* filtration was maintained. The theoretical analysis of filtration curves, discussed in chapter 2, have suggested that P_6 values are developed by the least deformable subpopulation of cells in the suspension, whereas the initial pressure rise of the filtration curve reflects deformability of the whole population of cells at the filter in the first instance (Nash, 1988b; Skalak, 1983). Thus the P_{grad} measurement would be more analogous to the INS. Also, the *in vivo* sequestration of neutrophils may be more sensitive to the changes in cell deformability than *in vitro* filtration measurements. Moreover, the lower INS measured for the two stable COPD patients than predicted for the P_6 values (Figure 3.4b) could reflect a reduced resistance to neutrophil lung transit due to changes in the pulmonary vascular bed in emphysema, as well as the deformability of the cells. Indeed these two subjects had more severe emphysema, as assessed by computed tomography scanning, than the rest of the patients in the group (Selby, 1991a).

Neutrophils from patients presenting with an exacerbation of COPD were consistently less deformable than neutrophils sampled from the same patient when in a clinically stable condition (Figure 3.6). This paralleled the increase in the initial sequestration of neutrophils measured in the lungs of COPD patients during exacerbations, and subsequently repeated when clinically stable when neutrophil sequestration was reduced (Selby, 1991a). Although the improvement in neutrophil deformability may have been due to the patients medication, this does not diminish the influence of cell deformability has on neutrophil passage through the microvasculature. Indeed, studies in the dog found that endotoxin-induced neutrophil pulmonary sequestration could be attenuated by pretreating neutrophils with pentoxifylline (Welsh, 1988), a methylzanthine derivative which has been shown to decrease neutrophil deformability (Schmalzer, 1984), but not by infusion of pentoxifylline which suggested a direct effect on cell deformability. However, the influence of cell-cell adhesion cannot be ignored, particularly in these inflammatory conditions. Indeed, as well as enhancing neutrophil deformability (Schmalzer, 1984), pentoxifylline was observed to decrease neutrophil adhesion to endothelial cells (Sullivan, 1984) which would also facilitate the passage of neutrophils through the pulmonary microcirculation.

3.5.5 CONTROVERSY OVER NEUTROPHIL ISOLATION AND RADIOLABELLING

It is possible that the reduced deformability and enhanced INS which was demonstrated in patients with an exacerbation of COPD (Selby, 1991a) may be caused by cell handling during the isolation procedure, resulting in activation of the cells already primed by the acute exacerbation. In the first studies using radiolabelled leucocytes, the isotope di-isopropylfluorophosphate ($DF^{32}P$) was employed which allowed labelling of the whole granulocyte pool *in vivo* following intravenous injection, and thus had the clear advantage of no *ex vivo* cell handling (Cartwright, 1964). However, the radiation burden of this label was high and, as cells with a long life span within the body were labelled, it posed a potential risk of mutagenesis. Moreover, for kinetic measurement of cells on their first passage through the lungs a bolus reinjection of the radiolabelled cells is necessary. The indium-oxine complex, used in the present studies, labels all blood cells (Thakur, 1977) and therefore necessitates isolation of pure neutrophil populations, particularly as the transit of neutrophils is compared with that of erythrocytes for these kinetic studies. Moreover, any differences which may exist between leucocyte subpopulations would be masked by labelling a mixed population of cells. Peritoneal neutrophils, as utilised by Rinaldo and associates (1988) in rats, although requiring minimal *ex vivo* cell handling, is not an option for human studies. Moreover, the response of neutrophils to a chemotactic stimulus, and their migration out of the vasculature into the peritoneum is likely to activate the cells. Indeed 75% of peritoneal neutrophils, following exposure to hypotonic lysis to remove contaminating erythrocytes, were retained on their first pass through the lungs (Rinaldo, 1988). In contrast, the cell isolation technique employed in the present studies, which also involves a hypotonic lysis step, measured a mean first pass neutrophil retention of only 5.6% in normal subjects (MacNee, 1989d) and 13.7% (or 0.17 INS) in normal elderly subjects (Selby, 1991a), Table 3.3 respectively). Thus for the *in vivo* neutrophil kinetic studies it was necessary to isolate pure neutrophil populations from peripheral blood. The controversy that exists over which harvesting procedure is least harmful has already been discussed (section 2.1.1). However, the condition of the isolated neutrophils is particularly relevant in these studies where the cells are reinjected, not least with regard to the safety of the subjects. In addition, there is an ongoing debate regarding the procedure for radio-isotope-labelling of cells necessary for these *in vivo* kinetic studies.

Questions have been raised about the viability and biological function of neutrophils labelled with oxine, the isotope vehicle used to label neutrophils in these studies

(McAffee, 1984; Thakur, 1984). Peters (1985b), Saverymuttu (1983a & 1985) and also Danpure (1986) discourage the use of oxine as the carrier molecule for ^{111}In. They recommend tropolonate as a carrier molecule which has the advantage of allowing cells to be labelled in the presence of plasma. The high affinity of plasma transferrin for the indium-oxine complex (Datz, 1985) necessitates the removal of plasma, which may lead to cell damage (Talstad, 1971). There are conflicting reports in the literature of reduced random motion, chemotaxis, and phagocytosis by ^{111}In-oxine labelled neutrophils in some studies (Burke, 1982; Saverymuttu, 1983a; Segal, 1978), whereas others report no effect on cell viability, ultrastructure, or function including random migration, chemotaxis and bacterial activity (Datz, 1985; Gunter, 1983; Hall, 1982; Zakhireh, 1979). Rinaldo (1988), although disapproving of any isolation procedure to obtain pure neutrophils, used ^{111}In-oxine for their *in vivo* kinetic studies. Tropolonate has also been demonstrated in some studies to be toxic to neutrophils (Gunter, 1983; Hall, 1982). However, using intravital microscopy, Lien and colleagues (1987b) observed that ^{111}Indium-tropolonate labelled neutrophils behaved the same as normal, unlabelled neutrophils in the pulmonary circulation. The most conclusive studies are those which have compared both oxine and tropolonate labelling of indium neutrophils. These studies have found no advantage for either binding agent for *in vivo* investigations of neutrophil influx into the lungs, or assessment of pulmonary microvascular injury (Das, 1988; Datz, 1985).
Despite the controversy which exists over the different techniques used to harvest and label neutrophils, some degree of neutrophil retention is observed whichever technique is used. A dissociation of the ^{111}In-label from the cells, and retention in the tissues, might account for the enhanced neutrophil lung transit, but is unlikely since less than 1% of the ^{111}In-label was found free in plasma.

The observation that neutrophils which have been subjected to an isolation procedure and a radiolabelling process are normally distributed within dog lungs (Hogg, 1988; Lien, 1987b), support the proposal that the sequestration is a normal physiological occurrence. Furthermore, the washout from the lungs of reinjected radiolabelled neutrophils, without immediate deposition in the liver (Selby, 1991a) confirms the functional integrity and survival of these neutrophils. The continuing circulation of neutrophils, which greatly exceeds their times of pulmonary transit, means it is unlikely that neutrophil survival (half life of 7 hrs)(Cartwright, 1964; Wintrobe, 1981) interferes with the first pass kinetics. Moreover, both in animal studies (Das, 1988; Haslett,1984) and in man (Datz, 1985; Selby, 1991a) radiolabelled neutrophils migrate to sites of inflammation and infection.

Furthermore, although the *in vitro* filtration of ^{111}In-labelled neutrophils demonstrated a slight increase in filtration pressures and hence reduced deformability compared with unlabelled cells, the pressures developed were not markedly greater to suggest cell activation as demonstrated in chapter 2.
Taken together these data suggest that neutrophil retention in the lungs is a physiological phenomenon and determined by cell deformability. The belief that neutrophil sequestration purely reflects cell damage, as proposed by Saverymuttu (1983a), is less likely. Moreover, the controversy does not detract from the major finding of a relationship between neutrophil sequestration in human lungs and cell deformability.

To conclude, the data demonstrate that upon reinjection the initial sequestration of leucocytes in the pulmonary vasculature is due to a limitation in deformability as they attempt to enter capillaries with diameters less than their own. This relationship was strengthened by observing an improvement in neutrophil deformability as patients recovered from an acute exacerbation of COPD, which paralleled a reduction in the INS as reported previously (Selby, 1991a). That the neutrophil 'washout' from the lungs did not correlate with the *in vitro* measurement of cell deformability suggests cell deformability was no longer a major determinant of lung microvascular transit once the cell had deformed and was within the capillaries. However, this is a complex signal, comprising recirculating and sequestered neutrophils. Indeed, further deformation could be required during passage of the capillary bed, such as at capillary stenosis (Bagge, 1976; Gaehtgens, 1984; Lien, 1990). Hence, cell deformability is likely to be an important determinant for each and every passage of neutrophils through the pulmonary microvasculature.

CHAPTER 4
THE EFFECT OF *IN VITRO* CIGARETTE SMOKE EXPOSURE

4.1 INTRODUCTION

Chronic cigarette smoking causes a blood (Cone, 1971; Corre, 1985; Hunninghake, 1983) and airway (Hunninghake, 1983; Rylander, 1974: Gadek, 1979; Stone, 1983) neutrophilia, which is associated with pulmonary dysfunction (Carel, 1988; Chan-Yeung, 1988; Richards, 1989). Such an influx of phagocytes into the peripheral airways may develop in response to complement activation by smoke itself (Kew, 1985; Robbins, 1991; Totti, 1984), or stimulated lung cells such as alveolar macrophages or epithelial cells (Gadek, 1980; Hunninghake, 1983). However, enhanced neutrophil sequestration in the lungs as a result of cigarette smoking may also occur prior to their activation by chemotactic stimuli. Neutrophils traversing the pulmonary microvasculature are only microns away from the external enviroment, thus placing them in prime position to respond to inhaled micro-organisms, but also subjecting them to inhaled toxins and pollutants such as cigarette smoke. Hence, although neutrophil sequestration may be initiated by reduced blood flow or increased adhesivity of the vascular endothelium, a direct effect of cigarette smoke on blood leucocytes cannot be ruled out.

Acute exposure to ozone, an oxidant present in many urban and industrial enviroments, results in airway inflammation, inclusive of an airway neutrophilia (Schelegle, 1991). The response to this oxidant is thus similar to the response seen with cigarette smoke inhalation, except that the changes are seen acutely following ozone inhalation, which to date has not been reported for acute cigarette smoke exposure. However, although not statistically significant, an increase in the circulating neutrophil count was observed in two separate studies following acute smoking (Anderson, 1991; Noble, 1975).

Buckley and colleagues (1975) were able to demonstrate evidence for the penetration of ozone through the alveolar tissue barrier by measuring oxidant-induced changes in peripheral blood erythrocytes and plasma. As this oxidant burden was determined after only a single, 2 hour 45 minute exposure to ozone, it is possible that components of cigarette smoke might also penetrate to the circulation, at least following chronic exposure if not acutely. Increased levels of COHb, and the presence of nicotine and cotinine in the blood of smokers is in support of this hypothesis (Benowitz, 1989). Moreover, morphological changes in rat aortic endothelium were found following acute exposure to fresh smoke (Pittilo, 1990 & 1984). Furthermore, increased alveolar epithelial permeability has been shown in man (Huchon, 1984; Jones, 1980; Kennedy, 1984; Mason, 1983), and animals (Minty, 1985; Mordelet-Dambrine, 1991; Witten, 1985) after cigarette smoke exposure, as

assessed by measuring an increased clearance of radiolabelled diethylenetriamine penta-acetate (DTPA), a low molecular weight chelating agent, from the lungs into the circulation. The mechanism by which smoking increases DTPA clearance from the lungs occurs is not as yet known, but it is possible that with increased lung permeability, increased penetration of components of smoke through the respiratory epithelium might also occur.

MacNee and colleagues (1989d) found that neutrophil sequestration in the lung microvasculature was enhanced during smoking. The possible mechanisms for this enhanced sequestration have been discussed in chapter 1. A reduction in neutrophil deformability appears to be a probable cause. Activation of neutrophils delayed within the lung microvasculature could then result in an excessive burden of oxygen radicals and proteases which have been implicated in the pathogenesis of smokers emphysema (Blue, 1978; Janoff, Carp, 1983).

In the previous chapter a strong correlation between neutrophil deformability, measured *in vitro* using a constant flow filtration system, and the sequestration of neutrophils on their first pass through the lungs was demonstrated. The aim of the studies in this chapter was to investigate the effect of smoke exposure on the deformability of neutrophils.

Several *in vitro* studies have observed the effects of whole or vapour phase cigarette smoke, or aqueous or condensate solutions of smoke on neutrophil function (Blue, 1978; Bridges, 1977; Corberand, 1980). Bridges and colleagues (1977) found that neutrophil chemotaxis was markedly altered by exposure to cigarette smoke *in vitro*, which was not due to cell cytotoxicity or depleted energy stores as oxidative metabolism was enhanced. A study by Corberand and associates (1979) similarly found a significant modification in neutrophil locomotion and oxidative metabolism following smoke exposure. They observed a significant reduction in the random and directional migration of peripheral blood neutrophils exposed to aqueous cigarette smoke. However, in contrast to the findings of Bridges et al (1977) decreased nitro blue tetrazolium (NBT) reduction suggested diminished oxidative metabolism. Unaltered oxygen consumption and phagocytosis was found in the same study (Corberand, 1979)

Although it is not surprising for cells to be functionally altered following exposure to smoke *in vitro*, or even *in vivo* in the mouth (Eichel, 1969; Eliraz, 1977) or bronchoalveolar space (Hoidal, 1981; Hunninghake, 1983; Rasp, 1978), altered function of peripheral blood leucocytes has also been reported following *in vivo*

smoking (Corberand, 1979; Noble, 1975). As with *in vitro* smoke exposure, *in vivo* smoke exposure produced a reduction in the random motion (Corberand, 1980) and chemotactic response (Noble, 1975) of peripheral blood neutrophils from smokers. Moreover, enhanced production of both the spontaneous and stimulated reactive oxygen intermediates by smoker's phagocytes has been reported (Cone, 1971; Corberand, 1979; Gillespie, 1987; Hoidal, 1982; Ludwig, 1982; Richards, 1989) which, together with increased neutrophil elastase activity in blood (Abboud, 1986; MacNee, 1989d), indicates activation of blood-borne leucocytes by inhalation of cigarette smoke. Thus these *in vitro* and *in vivo* studies suggest that neutrophil motility and oxidative metabolism are severely affected by cigarette smoke exposure.

For the work in this thesis, direct exposure to vapour phase cigarette smoke was chosen, although, in this chapter, the effect of cigarette smoke condensate on neutrophil deformability was also assessed. Moreover, neutrophil morphology, radical production and protease release were assessed following direct exposure to vapour phase cigarette smoke to determine the functional state of the cells.

4.2 AIMS

To determine the effect of acute vapour phase cigarette smoke or cigarette smoke condensate exposure on neutrophil deformability.

To assess the metabolic function and proteolytic activity of *in vitro* smoke exposed neutrophils.

4.3 MATERIALS AND METHODS

REAGENTS

All reagents were obtained from Sigma Chemical Company (Poole, UK) unless stated otherwise. Stock solutions of PMA, and fMLP were prepared as described in section 2.3. Stock solutions of CD18Mab (Dako Ltd., High Wycombe, UK), nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were prepared by dilution in PBS and stored at 4°C. CD18Mab was used within 1 week. Cigarette smoke condensate (CSC: WD & HO Wills, Imperial Tobacco Ltd., Hartcliffe, Bristol, UK) in DMSO was stored at -70°C and used within 1 week.

NEUTROPHIL HARVESTING

Neutrophils were harvested from venous blood sampled from healthy non-smokers as detailed in section 2.3.1.

IN VITRO SMOKE EXPOSURE

Two millilitres of the neutrophil suspensions ($1x10^6.ml^{-1}$ in PBS containing 0.5% BSA, unless stated otherwise) were exposed to vapour phase cigarette smoke generated by a smoking machine, as described previously (section 2.3.4). Control samples were sham exposed, under identical conditions but in air.

4.3.1 ASSESSMENT OF NEUTROPHIL DEFORMABILITY FOLLOWING *IN VITRO* SMOKE EXPOSURE

(a) CONSTANT FLOW FILTRATION

Neutrophil suspensions were exposed to 1, 3 or 5 puffs of vapour phase cigarette smoke delivered over 4 minutes in the tonometer system and the deformability of the population of neutrophils assessed using the constant flow filtration system as detailed in section 2.3.3.

(b) CELL TRANSIT ANALYSER (CTA)

The deformability of individual neutrophils was measured using a CTA (ABX Instruments, Montpelier, France)(Moessmer, 1990). The CTA, illustrated in Figure 4.1, consists of a fixed membrane centred between two reservoirs with the cell suspension on one side and a buffer solution on the other. A difference in the level of these two fluids creates a pressure gradient which causes the cell suspension to flow through the membrane. Membranes (ABX Instruments) with pores of nominally 5 μm or 8 μm diameters (manufacturers specification, although, the 8 μm pores were

measured to be 7.4 ± 0.1 μm (Buttrum)) were used, with a hydrostatic driving pressure of 8 or 18 cm H_2O respectively. The membrane was sonicated between each filtration to remove any residual cells or protein.

An electric current was applied across two electrodes, placed one in each fluid reservoir of the CTA, to monitor the change in electrical resistance which occurred as cells passed through the membrane. The pulses were digitised and analysed using an Archemides A310 computer (Watford Electronics Ltd, Watford, UK)(Fisher, 1992). Various parameters can be obtained from this pulse, such as the rise and fall time, threshold width, height, and transit time (Figure 4.2). Three further parameters are calculated from the first derivative of the digitised signal, i.e. the rise and fall rates, and the peak-to-peak width. The rise and fall rates are the maximum gradients of the upstroke and downstroke of the pulse respectively. The peak-to-peak measurement is the interval between the points of the maximum and minimum gradient of each pulse (Fisher, 1992). The data collected for each parameter for a sample are sorted into a histogram and the mean, median and cumulative percentiles for the distribution calculated. The number of valid pulses, as a percentage of the total number of pulses, is also recorded as the efficiency of the data collected.

Those parameters principally of interest in the present study were the peak-to-peak width, to give a measure of pore transit time, and the efficiency of pulse accumulation which reflects filter plugging by cells. The peak-to-peak parameter is believed to be a better measure of pore transit as this measurement is less susceptible to variation than the 'transit-time' parameter. Valid pulses are those with the four events - rise from baseline, attainment of plateau, fall from plateau, and return to baseline - occurring in the correct order. Two pulses colliding, or a pulse not returning to the starting baseline level results in rejection of those pulses.

For the smoking studies, pure neutrophils in PBS/0.5% BSA were exposed to 1 or 5 puffs of vapour phase cigarette smoke, diluted to a concentration of $1 \times 10^5.ml^{-1}$, and filtered immediately in the CTA. As a comparison, aliquots of control neutrophils (at $1 \times 10^5.ml^{-1}$) were exposed to 10^{-7}M fMLP and the pore transit time and efficiency of pulse collection determined as soon as the CTA could be loaded (approximately 30 seconds).

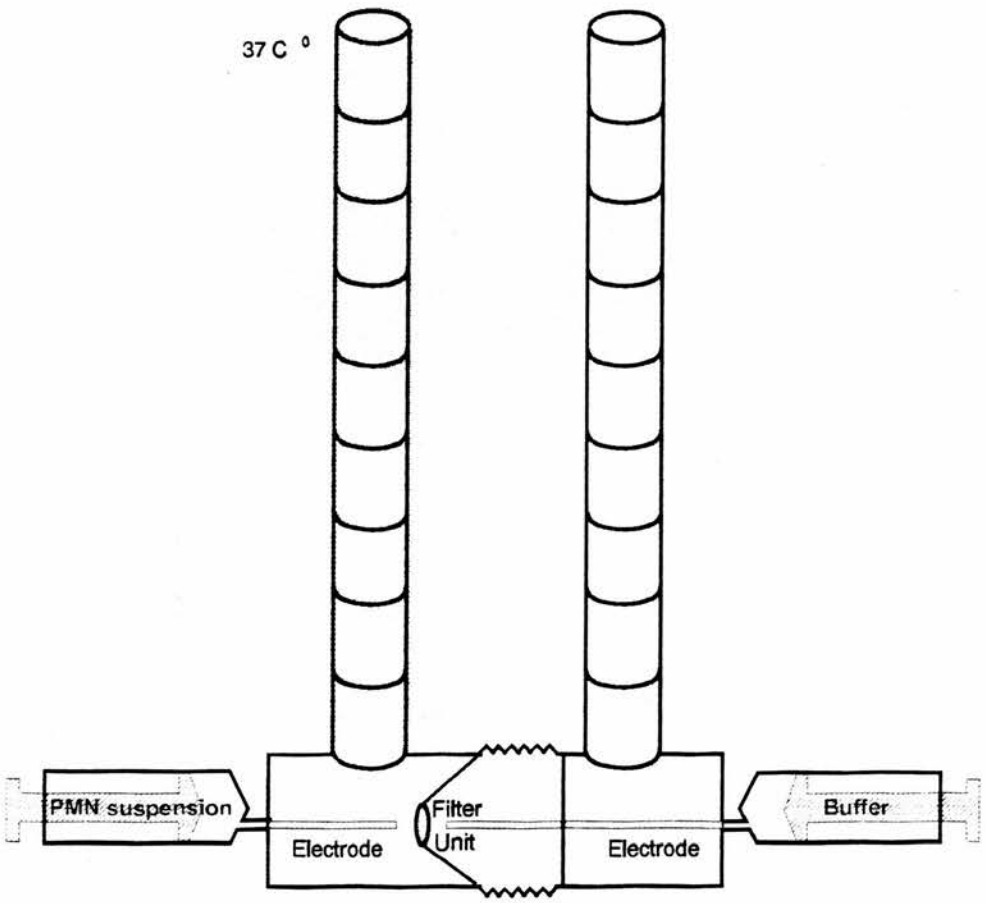


FIGURE 4.1
Schematic illustration of the Cell Transit Analyser (CTA). Neutrophil suspensions are placed in a reservoir which was maintained at 37°C (shaded area), with buffer in the reservoir on the other side of a micropore membrane. A pressure created by a difference in height between these two fluids causes the cell suspension to flow through the membrane. Cell transit through the membrane pores is detected as a change in electrical resistance placed across the membrane.

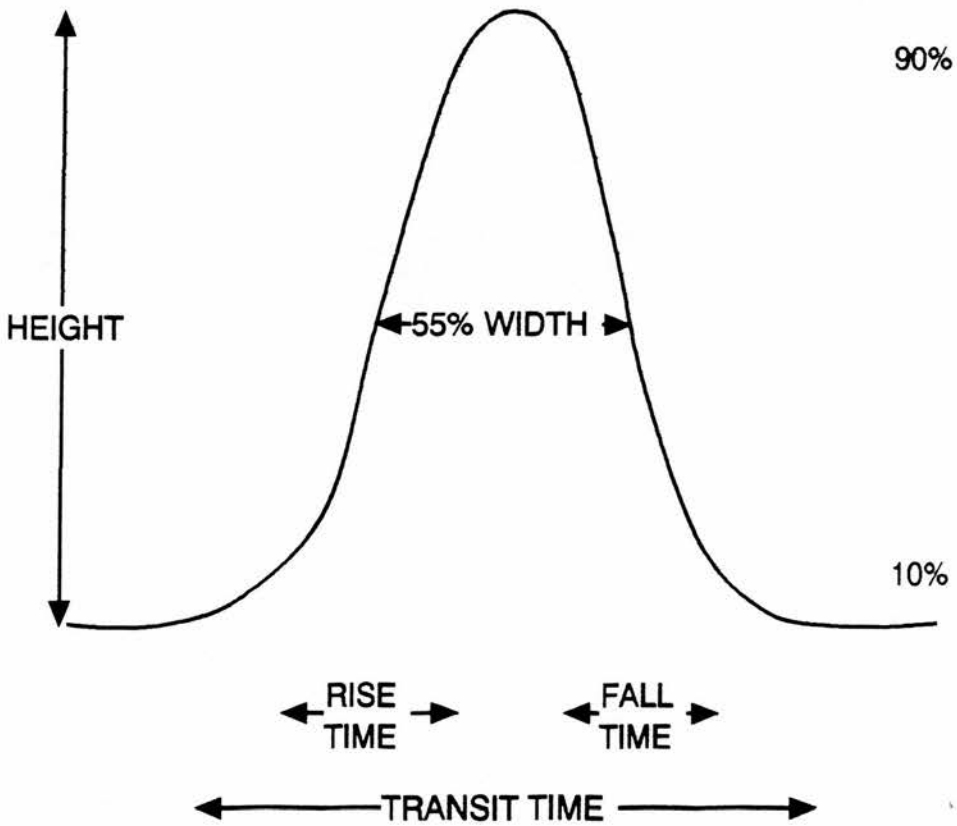


FIGURE 4.2
Diagram of a pulse obtained for cell transit through the membrane in the CTA illustrating the parameters which can be calculated (for details see text). (From Fisher (1992)).

(c) MICROPIPETTE ASPIRATION OF SMOKE EXPOSED NEUTROPHILS

Individual neutrophil deformability was also assessed by measuring cell entry times into various sizes of micropipettes as previously described (Paulitschke, 1993). Neutrophils were aspirated into capillary sized glass pipettes mounted on a micromanipulator (Narishige Co. Ltd., Tokyo, Japan) on an inverted light microscope (Leitz, Labovert, Germany)(Figure 4.3). Progress of the experiment was viewed on a Personal monitor (Philips), via a Hitachi CCTV video camera, and real time recordings were made possible by the addition of a Panasonic video recorder. A constant current was applied across the pipettes by electrodes in the chamber containing the cell suspension and in a hydrostatic pressure system. A negative aspiration pressure was imposed on the micropipette by fluid connection to this sensitive pressure system, and cell entry times were detected as a change in the conductance signal across the pipette as cells entered. The pulses were digitised and analysed using a BBC 'B' computer.

Deformation tests were performed on harvested neutrophils, at a final concentration of $1 \times 10^5.ml^{-1}$ suspended in PBS with the addition of 5% autologous plasma, held in glass chambers on the microscope stage. To begin the test the threshold pressure was established by adjusting the pressure until a cell remained stationary at the entrance to the pipette, without further flow. A negative pressure was then applied such that control neutrophils had a mean entry time of approximately half a second. The threshold pressure was re-checked at the end of each sample. Cells which took longer than 60 seconds to enter were expelled from the pipette and recorded manually as having a greater than 60 second entry time. Single, randomly selected neutrophils, following sham or smoke exposure, were aspirated into micropipettes with internal diameters of 4.3, 4.7, 5.5, 6.0, 6.7 or 8.4 μm using pressures of 12.5, 12.5, 5.5, 5.5, 2.0 cm H_2O or 5 mm H_2O respectively. Neutrophil entry times following fMLP stimulation (10^{-7}M for 20 minutes) into 4.5, 6.4 or 7.4 μm micropipettes using 10, 2, or 1 cm H_2O of pressure respectively, were also determined.

The CTA and micropipette experiments were performed in the Department of Haematology, The Medical School, Birmingham courtesy of Professor J Stuart and Dr GB Nash.

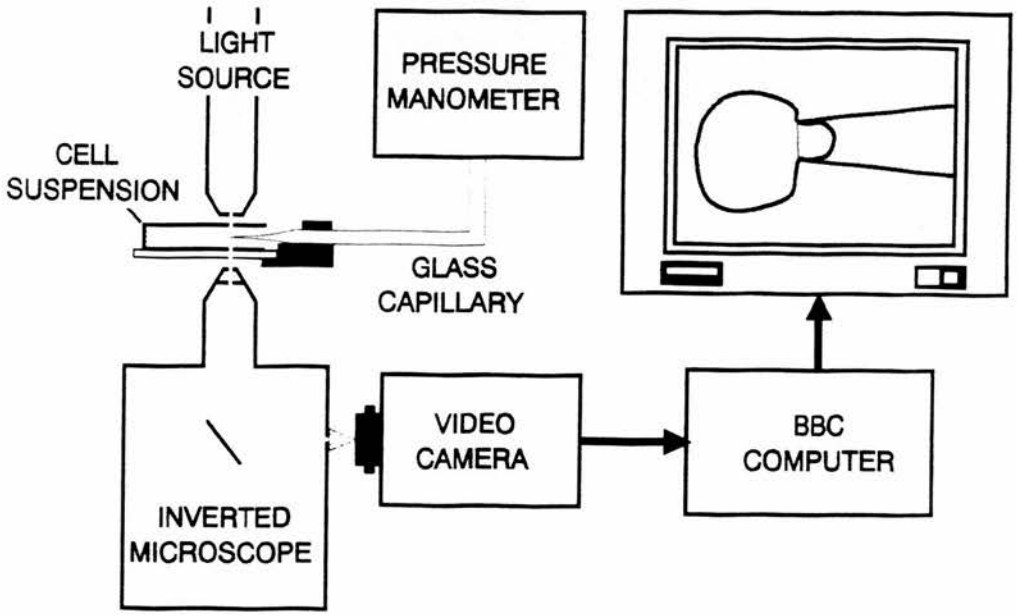


FIGURE 4.3
Schematic illustration of the micropipetting apparatus. Cell suspensions held in glass chambers are placed on the stage of an inverted light microscope. Glass micropipettes mounted on a micromanipulator are introduced into the suspension chamber and individual neutrophils are aspirated by applying a negative pressure to the pipette using a hydrostatic pressure system. Cell entry times into the pipette are detected by a change in resistance across the pipette.

4.3.2 THE INFLUENCE OF CD18-MEDIATED CELL ADHESION ON THE FILTERABILITY OF NEUTROPHILS

To determine whether the filterability of smoke exposed neutrophils was affected by cell adhesion to the micropore membrane, neutrophils in PBS ($1 \times 10^6.ml^{-1}$) were pre-incubated with the monoclonal antibody to the neutrophil integrin CD18 (CD18Mab; 1:60 dilution)(Dako Ltd., High Wycombe, UK) (Hogg, 1989). Following 30 minutes incubation at 22°C, excess antibody was washed off, and the cells resuspended in PBS/0.5% BSA at $1 \times 10^6.ml^{-1}$. Control and CD18Mab treated neutrophils were sham or smoke exposed, or PMA ($1\ \mu g.ml^{-1}$) stimulated, and immediately filtered using the constant flow filtration system.

4.3.3 THE EFFECT OF CIGARETTE SMOKE CONDENSATE ON NEUTROPHIL DEFORMABILITY AND ELASTASE RELEASE

Cigarette smoke condensate ($40\ mg.ml^{-1}$ stock) was kindly prepared by WD & HO Wills (Imperial Tobacco Ltd., Hartcliffe, Bristol, UK). The whole smoke from 2 cigarettes was condensed onto the sides of a glass vessel, and removed by washing with DMSO (Swain, 1969). Although the initial radical activity of cigarette smoke condensate is thought to be lost within the first 24 hours, the remaining activity is suggested to be stable, and relatively constant for up to one week when stored at -70 °C (manufacturers information). The condensate was, therefore, used within 1 week of preparation.

Neutrophil deformability was measured immediately following 4 minutes incubation in the tonometer with 1, 5 or 10% CSC, to mimic the smoke exposure conditions. Control samples were incubated in the presence of an equal concentration of DMSO.

Immunoreactive elastase levels in neutrophils and cell supernatants were measured, as detailed in chapter 2, following either 4 minutes exposure to 1% CSC (pH 7.4) in the tonometer, to mimic the smoke exposure conditions, or 30 minutes incubation at 37°C with 10% condensate to reproduce the original experiment by Blue and Janoff (1978). Control samples were incubated for a similar period with an equivalent concentration of DMSO.

4.3.4 THE EFFECT OF NICOTINE ON NEUTROPHIL DEFORMABILITY

To investigate whether the nicotine content of cigarette smoke altered cell deformability, nicotine at 1.85, 18.5 and 185 μM concentrations in PBS (made up fresh before use: pH 7.4) was added to neutrophil suspensions in the tonometer for 4 minutes. Cell deformability was assessed immediately following exposure using the positive pressure filtration system. Control samples were likewise rotated in the tonometer for 4 minutes, but in the presence of PBS alone.

4.3.5 FUNCTIONAL ASSESSMENT OF SMOKE EXPOSED NEUTROPHILS

(a) ASSAYS FOR REACTIVE OXYGEN INTERMEDIATES

Neutrophils in PBS/BSA were exposed to 5 puffs of vapour phase cigarette smoke, or sham exposed in air for 4 minutes. The cells were washed by centrifugation at 1100 rpm for 10 minutes, and the pellet resuspended in PBS to a concentration $10^7.ml^{-1}$. The spontaneous and PMA stimulated (0.1 and 1 $\mu g.ml^{-1}$) hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) release was assessed as described in section 2.3.2.

(b) NITRO BLUE TETRAZOLIUM (NBT) ASSAY

The NBT assay (Meerhof, 1986) was employed to measure endogenous oxidant production by neutrophils exposed *in vitro* to cigarette smoke.

NBT substrate solution was prepared by adding 300 μl of NBT stock solution (10 $mg.ml^{-1}$) followed by 33 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution (50 $mg.ml^{-1}$) to 0.1 M tris buffer containing 100 mM NaCl and 5 mM $MgCl_2$ (pH 9.5). Neutrophils (1×10^6 in PBS) were incubated with the NBT substrate solution (1 $mg.ml^{-1}$) for 15 min at 37°C. The cells were washed to remove excess NBT substrate, resuspended in PBS/0.5% BSA and exposed to 5 puffs of vapour phase cigarette smoke. Aliquots of untreated neutrophils and neutrophils following stimulation with 5 $\mu g.ml^{-1}$ PMA were used as negative and positive controls respectively. All samples were incubated for a further 15 minutes at 37°C and then cytospins (Shandon Cytospin; 500 rpm 5 minutes) were prepared onto glass slides. The cytospins were air dried, fixed and lightly counterstained using Dade Diff-Quick stain (Travenol Labs, Compton, UK). For each sample 100 cells were randomly selected and scored blind as formazan-negative, or weakly or strongly formazan-positive cells.

PROTEOLYTIC ACTIVITY OF SMOKE EXPOSED NEUTROPHILS

(a) IMMUNOREACTIVE NEUTROPHIL ELASTASE

Immunoreactive elastase release by neutrophils was determined by measuring the levels present in cell supernatants following sham or cigarette smoke exposure (2 ml at 1 x 10^6.ml^{-1} exposed to 5 puffs vapour phase cigarette smoke) by the method of Plow (1982) as described in section 2.3.2.

(b) FIBRONECTIN PROTEOLYSIS

Functional proteolytic activity was assessed by measuring the degradation of a protein matrix by sham and smoke exposed neutrophils. This assay was kindly performed by Dr GM Brown, Institute of Occupational Medicine, Edinburgh (Brown, 1988). Briefly, the method of McConahey and Dixon (1966) was used to label fibronectin in PBS with ^{125}I (Amersham International, UK) with an activity of 10,000 counts per minute in 100 ml. One hundred microlitres per well of the labelled fibronectin was dried onto microtiter plate wells by incubation at 37°C for 3 days. Sham and smoke exposed neutrophils (1 x 10^5 PMNL in F10 medium + 2% BSA)(F10, Gibco, Paisley), in triplicate, were cultured on the matrix for 4 hours at 37°C. Spontaneous and PMA (0.1 and 1 $\mu g.ml^{-1}$) stimulated proteolysis was assessed by measuring the ^{125}I counts present in 150 µl of the supernatant medium using a gamma counter (Rackgamma II, LKB, Finland). A background count in medium alone, and positive control consisting of trypsin (1 $mg.ml^{-1}$) in medium alone were also assessed.

MORPHOMETRIC ANALYSIS OF SMOKE EXPOSED NEUTROPHILS.

(a) QUALITATIVE ASSESSMENT

Neutrophil suspensions in PBS containing 0.5% BSA were washed following exposure to 5 puffs of vapour phase cigarette smoke, resuspended in PBS, and fixed by drop wise addition to 3% gluteraldehyde in PBS (pH 7.4). The fixed cells were washed once, resuspended in fresh gluteraldehyde and stored at 4°C until they could be processed as required for transmission and scanning electron microscopy (TEM and SEM respectively).

Following fixation with gluteraldehyde, the cells for SEM were post fixed with 1% osmium tetroxide in phosphate buffer. Dehydration was performed through graded ethanols to absolute at 30 minute intervals. critical point dried and mounted on stubs, sputter coated. The neutrophils were processed by the department of Human Genetics at the Western General Hospital, Edinburgh, viewed and photographed

with a scanning electron microscope by Dr B Walker, Pulmonary Research Laboratory, St Paul's Hospital, Vancouver, Canada.

(b) QUANTITATIVE MORPHOMETRIC ANALYSIS

The processing of neutrophils for TEM is described in chapter 2 (section 2.3.2). Two hundred cells each (from 6 subjects) of sham and smoke exposed neutrophils were measured from TEM photographs, using a Tektronix 4050 series computer linked to a digitising tablet (GIS Blairgowrie, Scotland). The measurements were performed by Susan Lannan. The minimum diameter, maximum diameter (at the perpendicular), circumference, and area of the cell were measured.

4.3.6 RECOVERY OF NEUTROPHIL DEFORMABILITY AND FUNCTION FOLLOWING CIGARETTE SMOKE EXPOSURE

To determine whether the effect of *in vitro* smoke exposure on neutrophils was reversible, the deformability and the generation of reactive oxygen intermediates were assessed after a recovery period of 1 hour.

RECOVERY OF NEUTROPHIL DEFORMABILITY

Neutrophil deformability was assessed immediately following smoke exposure, and after incubation at 22 °C in: (1) the same PBS containing 0.5% BSA in which the cells were exposed to smoke, (2) fresh PBS/ BSA; (3) PBS containing 3 μM GSH; (4) autologous plasma. The cells were recounted following incubation, and filtered at 1 x $10^5.ml^{-1}$ in the constant flow filtration system.

RECOVERY OF NEUTROPHIL OXYGEN RADICAL RELEASE

To assess the recovery of neutrophil function, smoke exposed neutrophils were washed and either resuspended in PBS and immediately assessed for their ability to release H_2O_2 and O_2^-, or resuspended in an equal volume of autologous plasma to allow the cells to recover. After incubation for 1 hour at 22°C, the autologous plasma was removed by centrifugation at 1100 rpm for 10 minutes. The cells were resuspended in PBS (1 x $10^7.ml^{-1}$) and the spontaneous and PMA stimulated (0.1 and 1 $\mu g.ml^{-1}$) oxygen radical release determined (detailed in section 2.3.2).

4.3.7 STATISTICAL ANALYSIS

Statistical analysis applied to the data in this chapter was as detailed in chapter 2. In addition, for the micropipette data a Kolmogorov-Smirnoff test was used to allow non-parametric comparison of the distributions for two treatments with uneven sample numbers (Snedecor, 1974).

4.4 RESULTS

4.4.1 NEUTROPHIL DEFORMABILITY FOLLOWING *IN VITRO* SMOKE EXPOSURE

(a) CONSTANT FLOW FILTRATION SYSTEM

A dose related decrease in neutrophil filterability was evident from a progressive increase in filtration pressures following 1, 3 or 5 puffs of cigarette smoke (Figure 4.4). Although a small but measurable increase in the filtration pressures occurred in all 7 samples after exposure to one puff of smoke, the mean difference was not significantly different from sham exposed neutrophils in comparison to the higher doses of smoke (Figure 4.4). The percentage COHb measured in similarly exposed venous blood increased with increasing dose of exposure (COHb: sham 1.1 ± 0.4%; 1 puff 2.4 ± 0.4%; 3 puffs 4.0 ± 1.0%; 5 puffs 6.5 ± 1.8%: n=5, $p<0.05$, $p<0.01$ and $p<0.01$ respectively).

(b) CTA

Pore transit times (peak-to-peak width parameter) for smoke exposed neutrophils through an 8 μm pore membrane in the CTA were not significantly different compared to control and sham exposed neutrophils (Figure 4.5a). Nor was the efficiency of pulse collection significantly different (efficiency: sham exposed 66.1 ± 8%, smoke exposed 60.6 ± 9.4%; n=9, $p>0.05$). In contrast, pore transit time for fMLP stimulated neutrophils was significantly altered , although from the percentiles of the transit time distribution it was evident that less than 50% of the population responded to fMLP stimulation (Figure 4.5b).

Pore transit times through a 5 μm pore membrane were markedly longer but not different for sham and smoke exposed neutrophils (median values for 2 subjects: sham exposed 52.5 milliseconds, smoke exposed 59.2 millisseconds). However, the efficiency of pulse collection was low (sham exposed 10.3%, smoke exposed 14.5%) with the majority of the pulses being rejected by the computer software.

(c) MICROPIPETTE ASPIRATION OF SMOKE EXPOSED NEUTROPHILS

Likewise, entry times for micropipette aspiration of neutrophils following fMLP stimulation were considerably longer than for control neutrophils (Table 4.1). A shift towards longer entry times was measured for smoke exposed neutrophils, compared with sham exposed cells, with a greater than 60 second entry time being recorded for a large proportion of the cells (Table 4.2). Data for the (a) 4.7 μm and (b) 6.7 μm pipettes are illustrated in Figure 4.6.

Even for a lower dose (1 puff) of smoke exposure, pipette entry times were greater than for control or sham exposed cells (Table 4.3). The slower pipette entry time for

smoke exposed neutrophils appeared to be due to the rigid membrane blebs (Table 4.4 and Figure 4.7).

4.4.2 THE EFFECT OF NEUTROPHIL ADHESION ON THE FILTERABILITY MEASUREMENT.

Pre-incubation of the neutrophils with CD18Mab did not alter the filtration pressures developed by sham, smoke exposed neutrophils (Figure 4.8a), or PMA stimulated neutrophils (Figure 4.8b) compared with cells not treated with CD18 Mab.

4.4.3 THE EFFECT OF CIGARETTE SMOKE CONDENSATE ON NEUTROPHIL DEFORMABILITY AND ELASTASE RELEASE

The addition of cigarette smoke condensate (CSC, 1% of stock) for 4 minutes in the tonometer had no effect on neutrophil deformability or human neutrophil elastase (HNE) release, compared with control neutrophils exposed to 1% DMSO (Table 4.5). Although an increase in neutrophil deformability was found for increasing condensate concentrations (control 4.93 ± 0.3 cm H_2O; 1% CSC 3.42 ± 0.9 cm H_2O; 5% CSC 2.5 ± 0.3 cm H_2O; 10% CSC 2.6 ± 1.1 cm H_2O: n=3), this was associated with decreasing cell viability (viability: control cells 98%; 1% and 5% CSC 93%; and 10% CSC 33%). Incubation with 10% smoke condensate for 30 minutes result in enhanced elastase release associated with a corresponding decrease in cell viability (Table 4.5).

4.4.4 THE EFFECT OF NICOTINE ON NEUTROPHIL DEFORMABILITY

Incubation of neutrophils with 1.85, 18.5, or 185 μM nicotine for 4 minutes in the tonometer did not affect cell deformability (P_6 control 5.0 ± 0.4 cm H_2O; 1.85 μM 5.7 ± 0.3 cm H_2O; 18.5 μM 6.1 ± 1.5 cm H_2O; 185 μM 5.1 ± 0.9 cm H_2O: n=5, $p>0.05$). The solutions were not cytotoxic to the cells as viability was maintained (control 98.7%, 185 μM nicotine 90.2%).

4.4.5 ASSESSMENT OF NEUTROPHIL FUNCTION FOLLOWING *IN VITRO* SMOKE EXPOSURE

OXYGEN RADICAL PRODUCTION BY *IN VITRO* SMOKE EXPOSED NEUTROPHILS

(a) EXOGENOUS RADICAL RELEASE

The spontaneous release of H_2O_2 and O_2^- was significantly reduced following *in vitro* smoke exposure (Figures 4.9a and b). Although an increased H_2O_2 and O_2^- release could be invoked from sham exposed neutrophils by stimulation with 0.1 and 1.0 $\mu g.ml^{-1}$ PMA, the levels generated by smoke exposed cells were not significantly enhanced by PMA stimulation and hence markedly lower than PMA stimulated, sham exposed neutrophils.

(b) ENDOGENOUS RADICAL PRODUCTION

The NBT assay did not indicate any change in endogenous radical production in neutrophils exposed to cigarette smoke, compared with sham exposed or control cells (Table 4.6). In contrast, enhanced radical production was observed for PMA stimulated neutrophils (Table 4.6).

PROTEOLYTIC ACTIVITY OF *IN VITRO* SMOKE EXPOSED NEUTROPHILS

(a) ELASTASE RELEASE

The levels of immunoreactive elastase in the cell supernatants from sham or smoke exposed neutrophils were not significantly different (sham exposed 1.8 ± 0.7 $ng.ml^{-1}$, smoke exposed 2.2 ± 0.7 $ng.ml^{-1}$, $n=6$, $p>0.05$) which contrasts the increase levels of elastase detected in the supernatants from PMA stimulated neutrophils (section 2.4.2)

(b) DEGRADATION OF A PROTEIN MATRIX

Spontaneous degradation of a fibronectin matrix was suppressed following smoke exposure in most samples studied (10/11), but statistical significance was not attained (Figure 4.10). PMA stimulation of neutrophils did not alter fibronectin proteolysis following either sham or smoke exposure (Figure 4.10).

MORPHOMETRIC ANALYSIS OF SMOKE EXPOSURE

Transmission and scanning electron micrographs of the smoke exposed neutrophils revealed changes in the neutrophil membrane in the form of blebs and ruffles which were not present in the sham exposed samples (Figures 4.11a and b). Morphometric assessment of neutrophils exposed to vapour phase cigarette smoke revealed an increase in the maximum diameter and circumference, but no change in the minimum

diameter and area ($p>0.05$), compared with sham exposed cells (Figure 11a)(Table 4.7).

4.4.6 RECOVERY OF SMOKE EXPOSED NEUTROPHILS

ABILITY TO DEFORM

An improvement in cigarette smoke exposed neutrophil deformability was observed following incubation for 1 hour in fresh PBS/BSA; PBS with GSH; or in autologous plasma (Figure 4.12). Plasma was the most effective treatment in ameliorating the smoke-induced reduction in cell deformability. The reduction in filtration pressures in these recovery experiments was not due to cell loss as each aliquot was recounted after smoke exposure and filtered at 1×10^5 cells.ml^{-1}.

OXYGEN RADICAL PRODUCTION

Recovery of neutrophil oxygen radical release was investigated by comparing the levels of H_2O_2 and O_2^- released from neutrophils following incubation for 1 hour in the presence of autologous plasma (Figures 4.13a and b) with the radical release from the same aliquot of cells assessed immediately following smoke exposure (Figure 4.9).

Whereas neutrophils exposed to smoke measured, as described previously, a reduction in both spontaneous and PMA stimulated H_2O_2 release (Figure 4.9), smoke exposed neutrophils incubated for 1 hour in autologous plasma showed recovery from this effect (Figure 4.13a). However, the levels of PMA stimulated release from these cells did not return to control levels when incubated in plasma.
Similar results were obtained for PMA stimulated O_2^- release from neutrophils exposed to smoke and allowed to 'recover' in plasma (Figure 13.b).

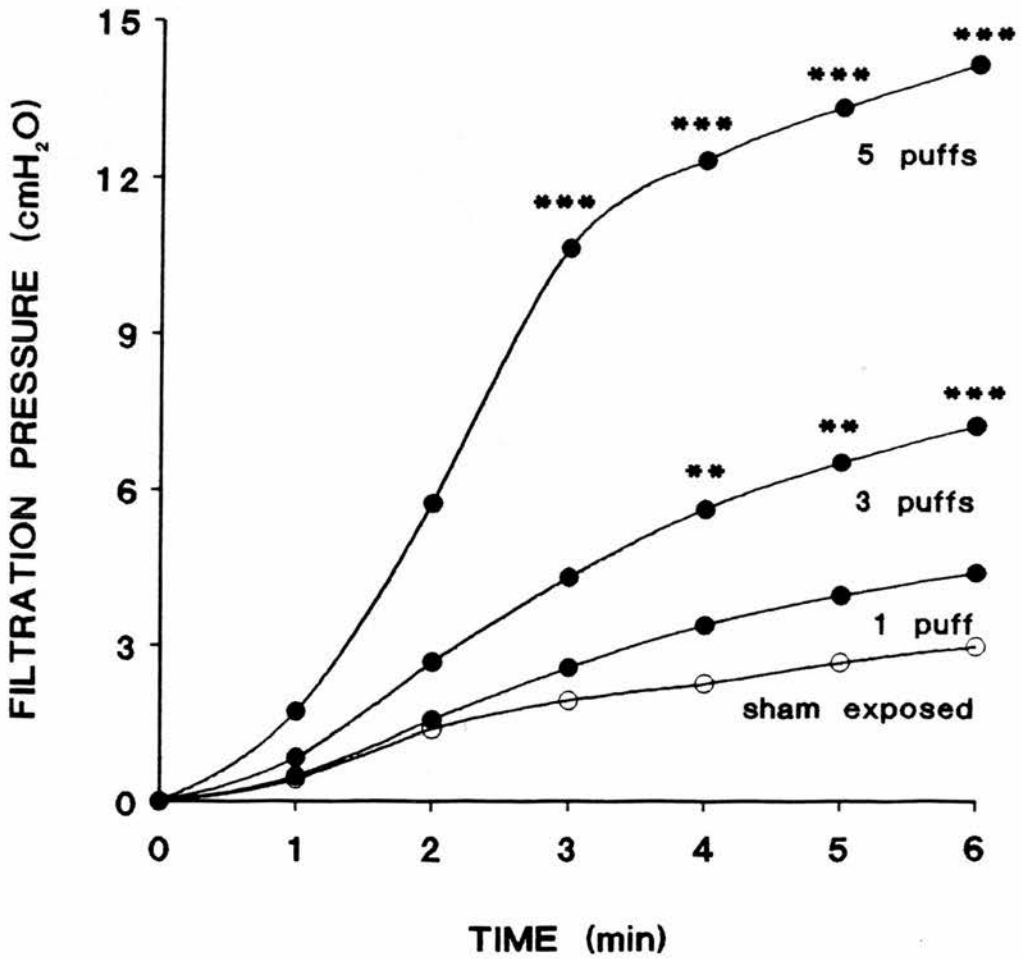
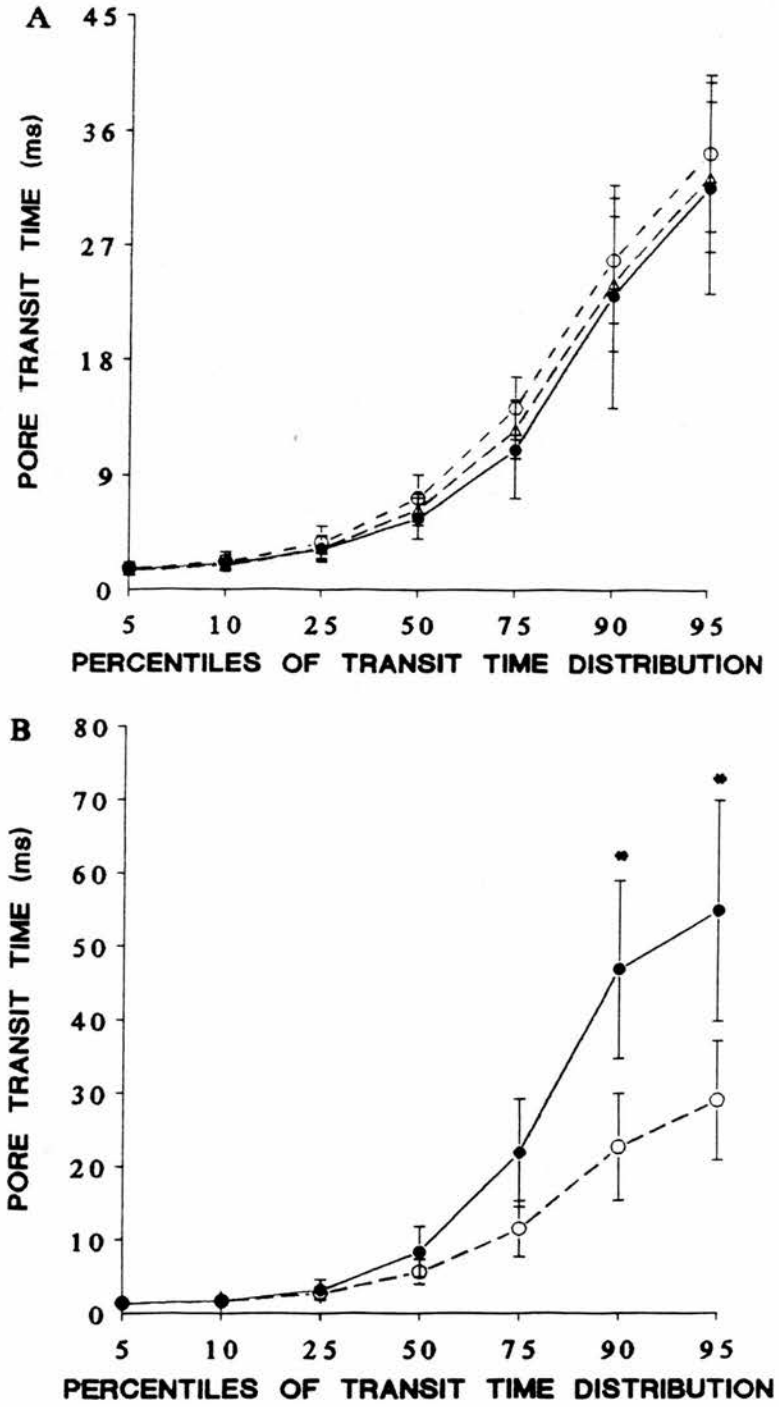
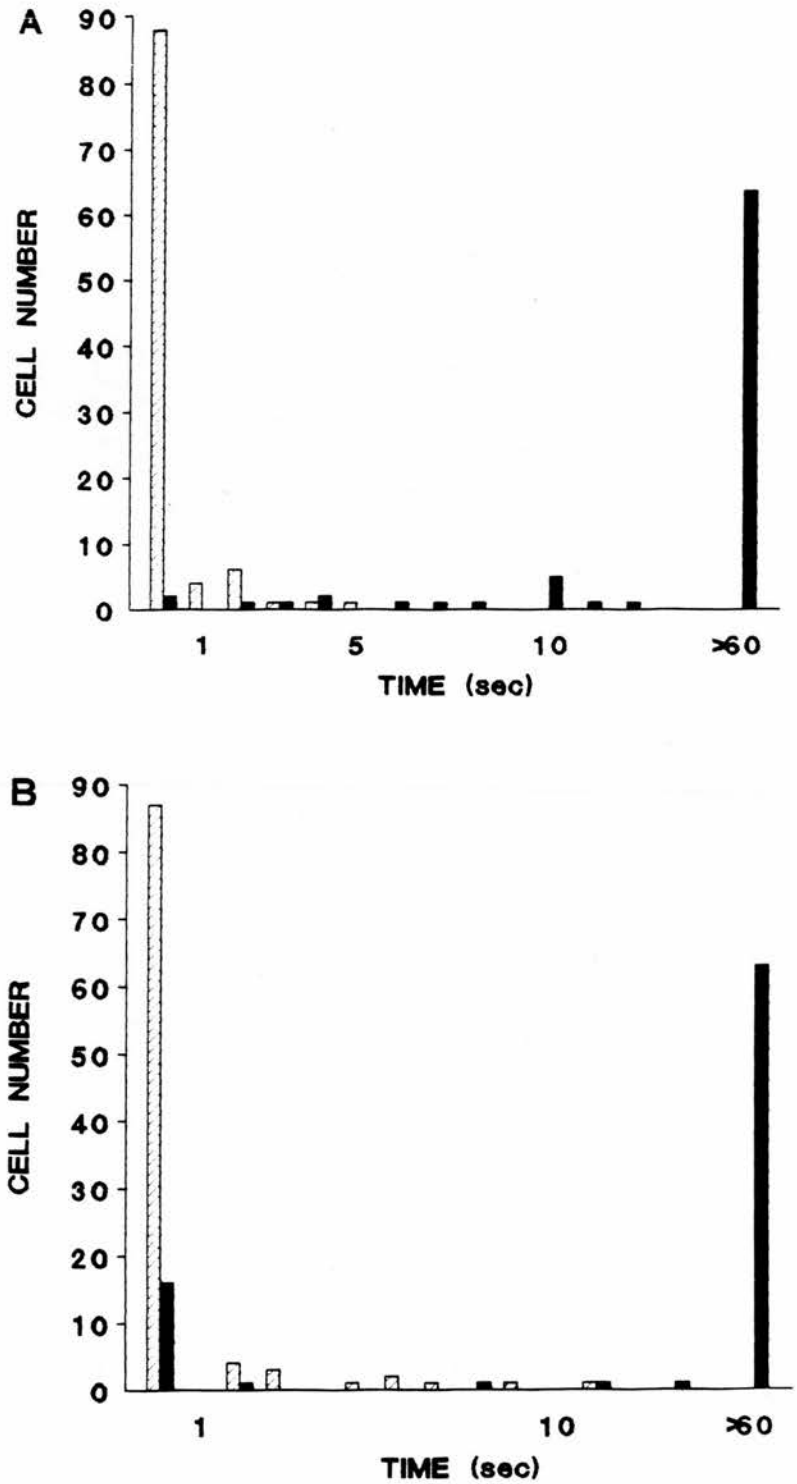


FIGURE 4.4
The effect of *in vitro* smoke exposure on neutrophil deformability measured using a constant flow filtration system. Neutrophils were exposed to 1, 3 or 5 puffs of vapour phase cigarette smoke. Smoke exposure (●) resulted in a dose dependent increase in filtration pressures compared with sham exposed neutrophils (O). Mean values for 5 experiments are shown. Error bars are not shown for clarity. ** $p<0.01$, *** $p<0.001$



FIGURES 4.5a and b
Cumulative percentiles of the transit time distribution for neutrophil passage through an 8 μm pore membrane in the Cell Transit Analyser. Transit times were measured for (a) control (O), sham (Δ) and smoke exposed neutrophils (5 puffs)(●) and (b) control neutrophils (O) and neutrophils immediately following fMLP stimulation (10^{-7}M)(●). Smoke exposure did not alter neutrophil pore transit (n=9, $p>0.05$). In contrast, fMLP stimulation increased the transit time for approximately half the cell population (n=5, $p<0.05$). Mean values are shown with error bars representing 1 SD.



FIGURES 4.6a and b
Frequency distribution of entry times for control (hatched bars) and smoke exposed neutrophils (5 puffs)(solid bars) into (a) 4.7 μm or (b) 6.7 μm diameter pipettes. An increase in pipette entry times was observed as a result of smoke exposure for all pipettes ($p<0.001$)(Tables 4.3 and 4.3).

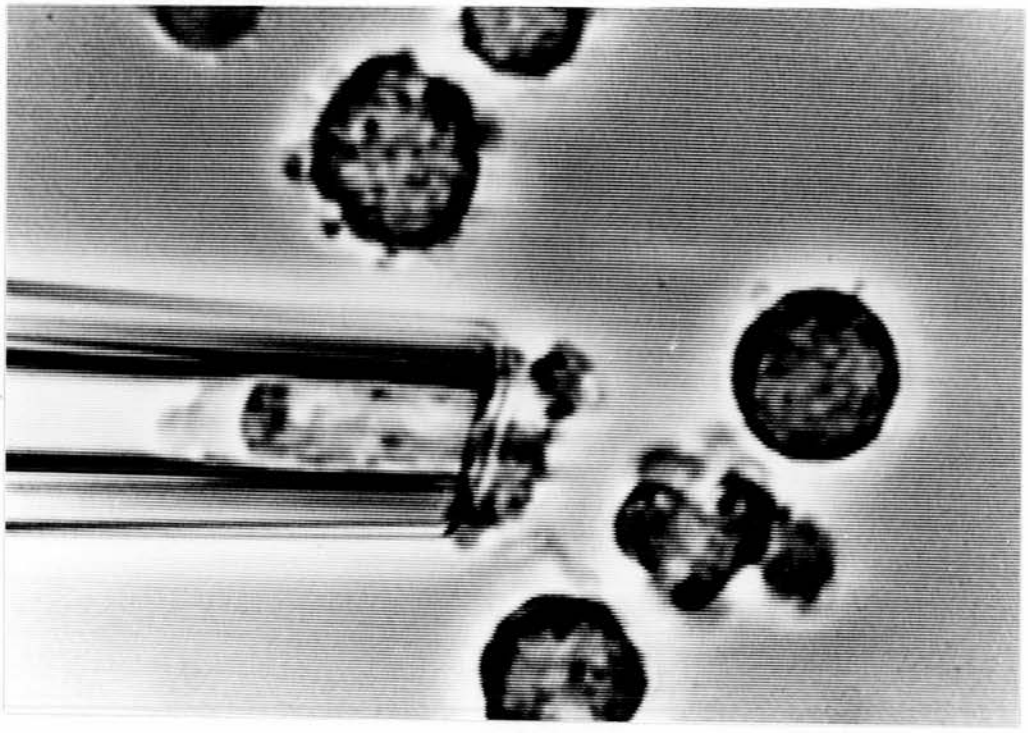
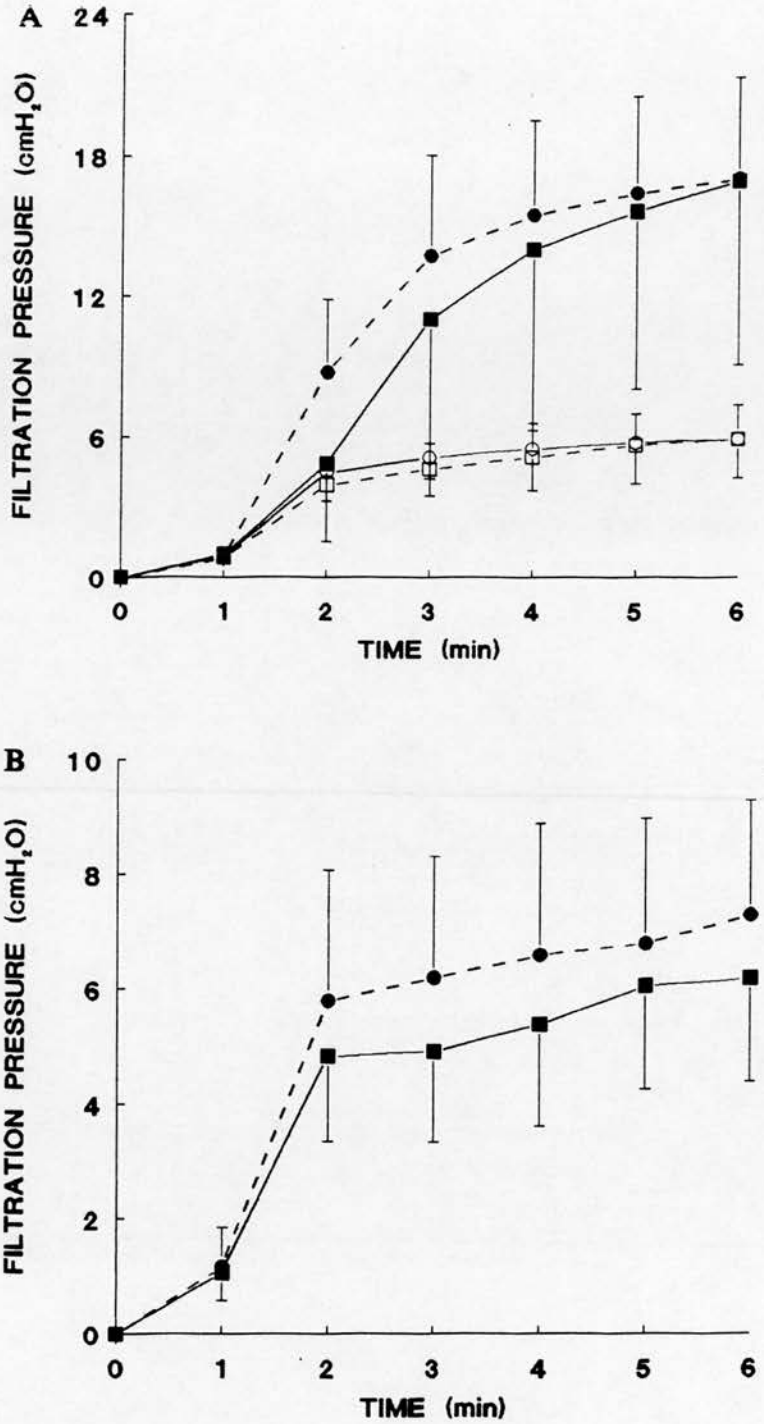
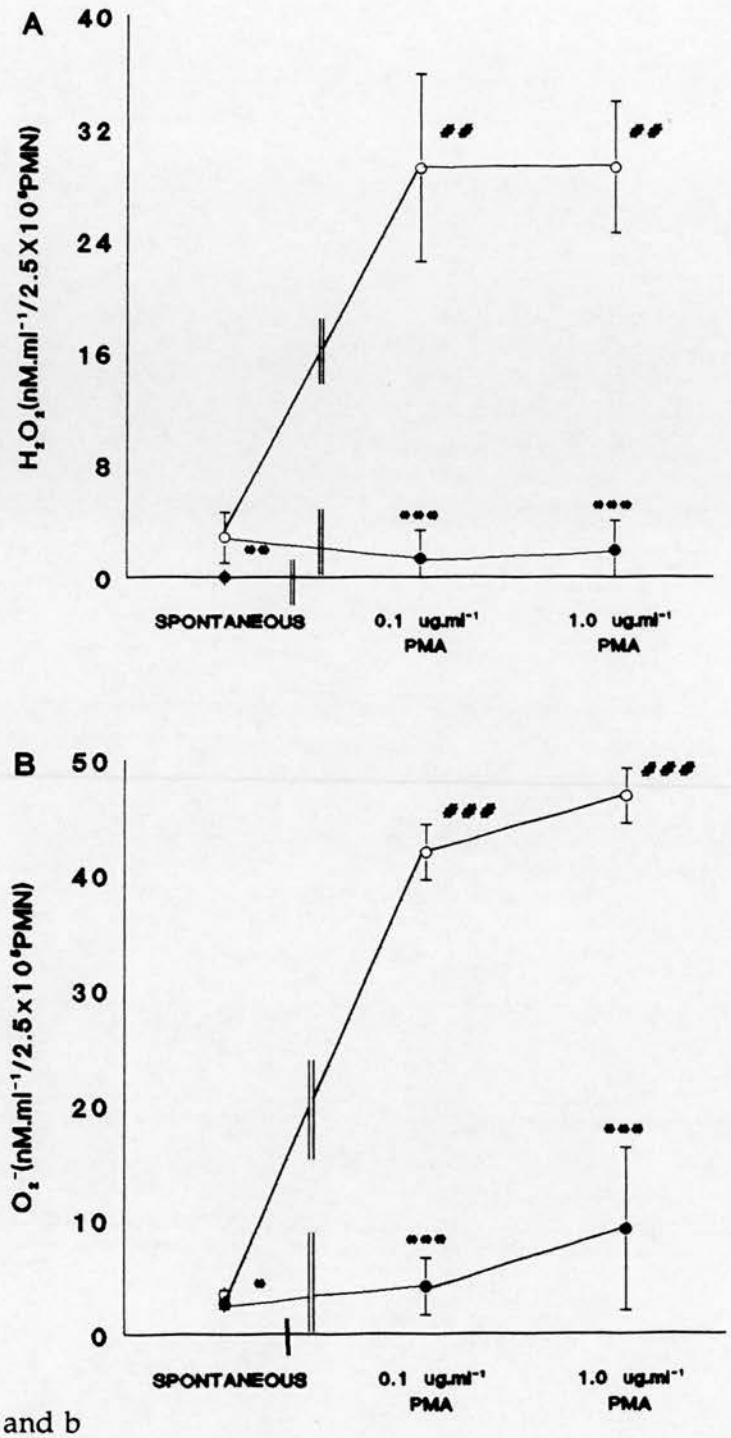


FIGURE 4.7
Photomicrograph of the entry of a smoke exposed neutrophil into a micropipette (diameter 4.7 μm). Neutrophils with blebbed and normal morphology are shown.



FIGURES 4.8a and b
The effect of preincubation with a monoclonal antibody to the neutrophil CD18 adhesion receptor (CD18Mab) on the filterability of (a) smoke exposed neutrophils (n=5)(●) and (b) PMA stimulated (1 $\mu g.ml^{-1}$)(n=4)(●) neutrophils. The filterability of neutrophils preincubation with CD18Mab (■) was not different from the filterability of smoke exposed or PMA stimulated neutrophils not pretreated with CD18Mab (●). Nor was the filterability of sham exposed neutrophils altered by preincubation with CD18Mab (Figure 4.8a, open symbols). Mean values are shown with error bars representing 1 SD.



FIGURES 4.9a and b

The release of (a) H_2O_2 and (b) O_2^- by sham (O) and smoke exposed (●) neutrophils. Radical release was measured over 2 hours immediately following *in vitro* smoke exposure. The spontaneous and stimulated release of H_2O_2 and O_2^- was significantly reduced by smoke exposure compared to sham exposed neutrophils. Whereas sham exposed neutrophils could be triggered to release more H_2O_2 and O_2^-, smoke exposed cells did not respond to stimulation. Mean and error bars representing 1 SD for 5 experiments are shown. Comparing smoke to sham exposed cells, *p<0.05, **p<0.01, ***p<0.001. Comparing stimulated to spontaneous release, #p<0.05, ##p<0.01, ###p<0.001.

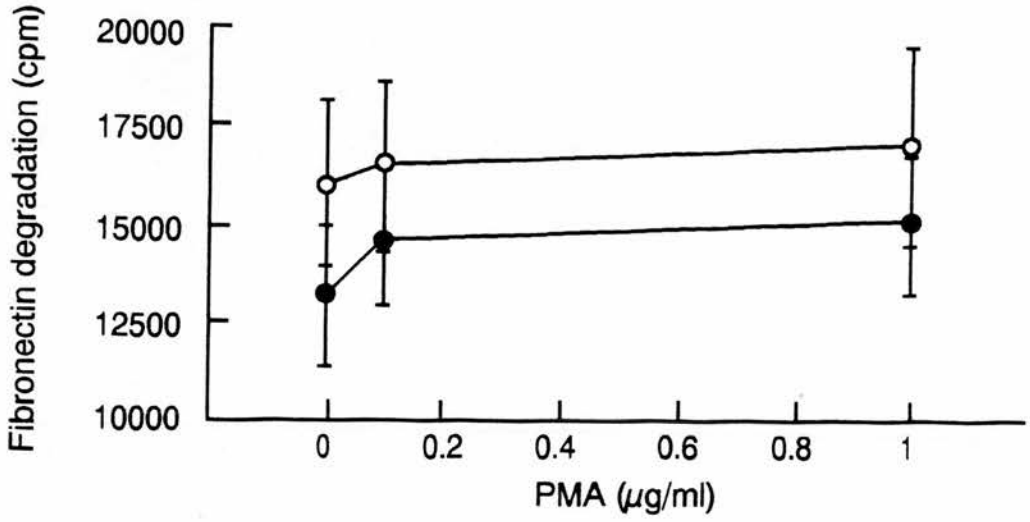
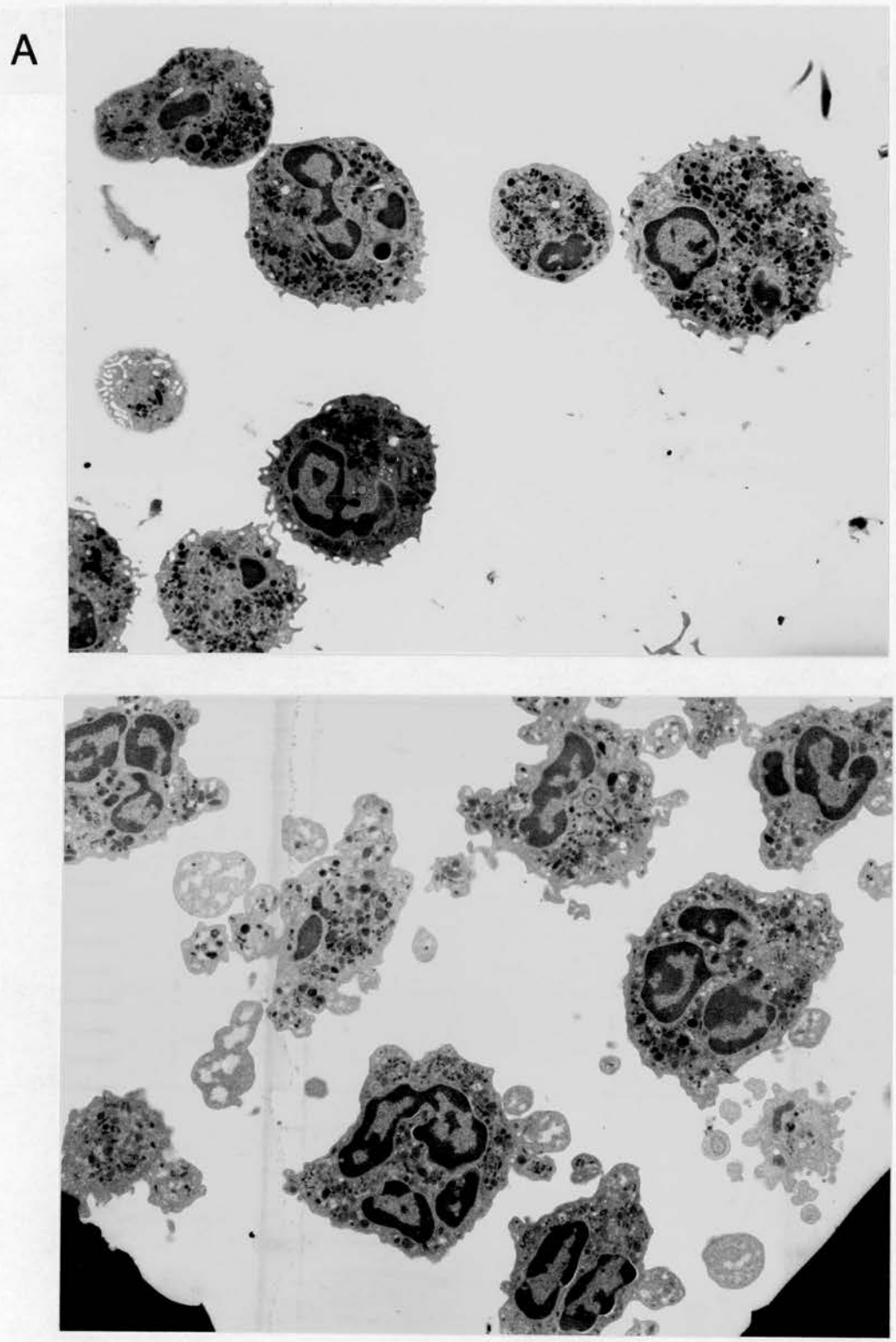


FIGURE 4.10
The effect of *in vitro* smoke exposure on fibronectin degradation by (O) sham and (●) smoke exposed neutrophils. Spontaneous and PMA stimulated (0.1 and 1.0 $\mu g.ml^{-1}$) fibronectin degradation was assessed over a 4 hour period. Degradation of the protein matrix by sham and smoke exposed neutrophils was not significantly different, although a reduction in the spontaneous degradation by smoke exposed neutrophils was noted for 10/11 experiments. Stimulation with PMA did not significantly increase fibronectin degradation by either sham or smoke exposed neutrophils. Mean values with error bars representing 1 SD are shown.



FIGURES 4.11a and b
Transmission (a) and scanning electron (b) micrographs of a sham (upper) and smoke exposed (lower) neutrophils. Compared to the normal spherical shape and membrane ruffles of sham exposed neutrophils, smoke exposed cells had changed shape and blebbing of the plasma membrane was evident. Magnification x4.2 x 10^3 and x9.2 x 10^4 respectively.

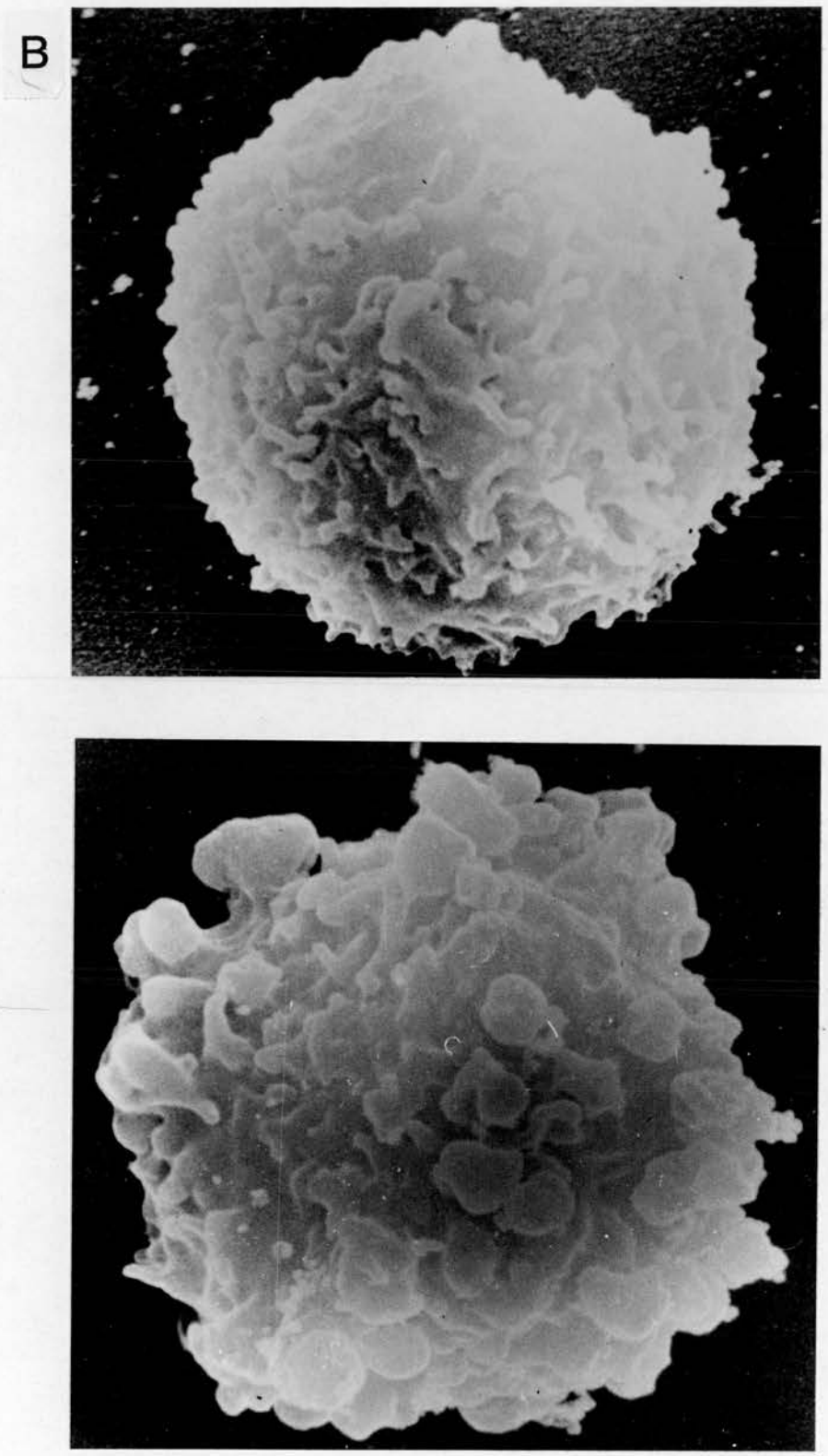


FIGURE 4.11 CONTINUED
Scanning electron micrograph of a sham (upper) and a smoke exposed neutrophil (lower).

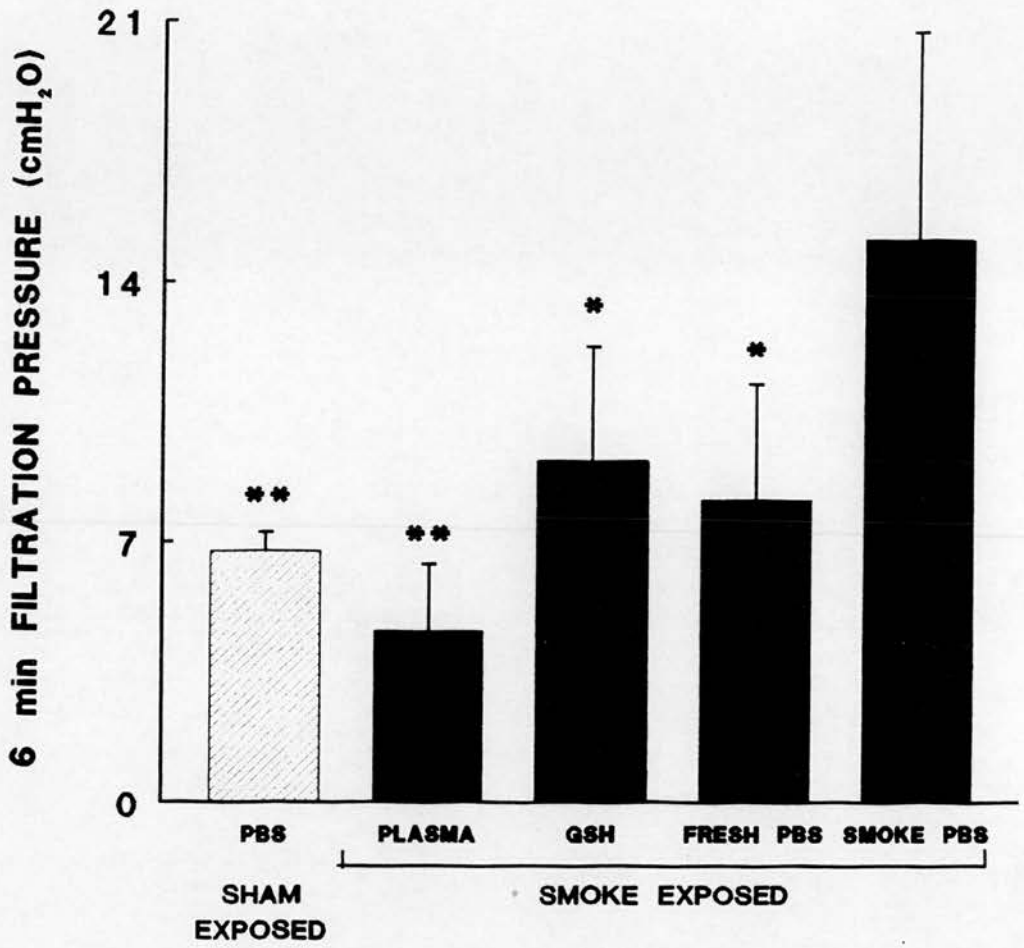


FIGURE 4.12
The pressures developed after 6 minutes filtration (P_6) for smoke exposed neutrophils (closed bars) allowed to recover for 1 hour in the presence of PBS/BSA in which they were exposed (smoke PBS); fresh PBS/BSA; 3 μM GSH; or autologous plasma. The pressures developed by smoke exposed neutrophils were increased compared with sham exposed neutrophils (hatched bars). Those resuspended in fresh PBS, GSH or plasma developed less pressure than those in smoke exposed PBS (P_6 PBS and GSH, $p<0.05$; plasma, $p<0.01$). Error bars represent 1 SD for 7 experiments.

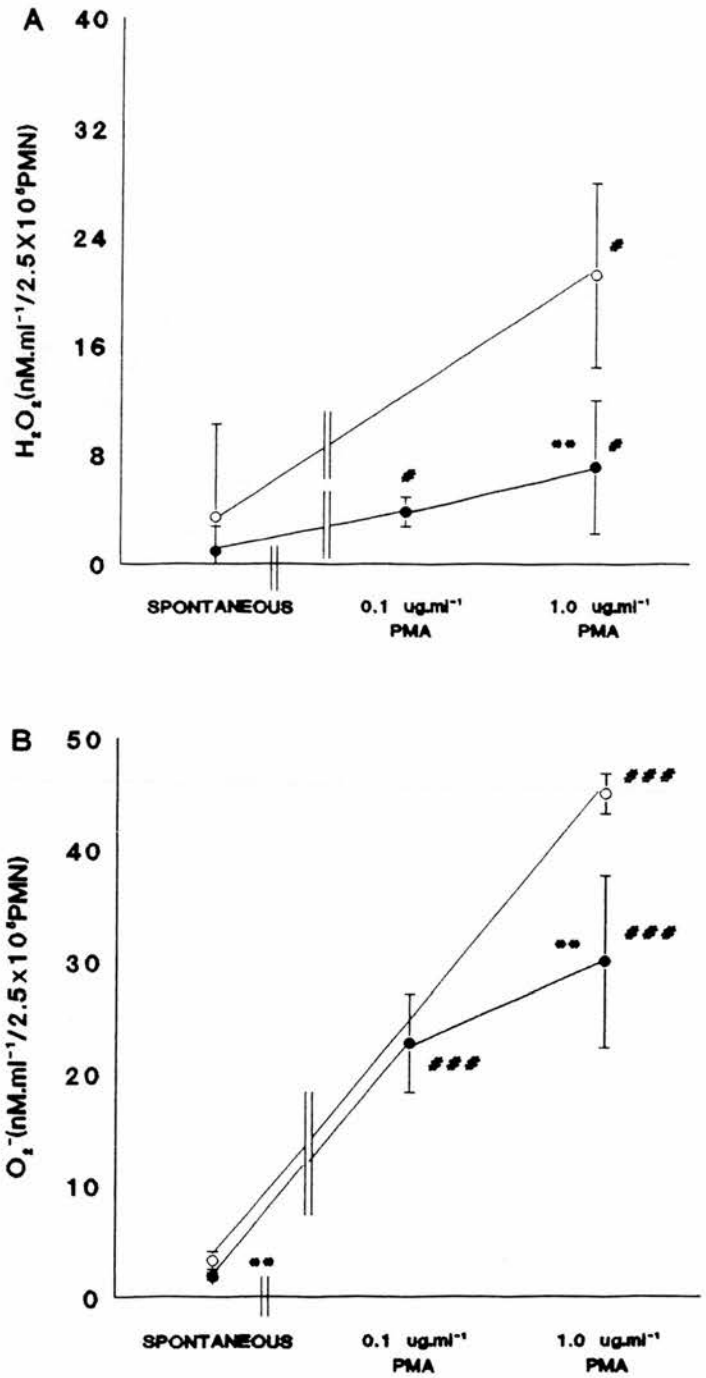


FIGURE 4.13a and b

The release of (a) H_2O_2 and (b) O_2^- by sham (O) and smoke exposed (●)neutrophils allowed to recover for 1 hour in autologous plasma. In contrast to the findings immediately following smoke exposure (Figure 4.9a), spontaneous H_2O_2 release from smoke exposed neutrophils was similar to that of sham exposed neutrophils. However, spontaneous O_2^- following incubation for 1 hour in plasma was still reduced. Incubation in autologous plasma enhanced the PMA stimulated release of both H_2O_2 and O_2^-, but the levels were still significantly less than for incubated sham exposed neutrophils. Mean and 1 SD for 5 experiments are shown. Comparing smoke to sham exposed cells, *p<0.05, **p<0.01, ***p<0.001. Comparing stimulated to spontaneous release, #p<0.05, ##p<0.01, ###p<0.001.

TABLE 4.1

MICROPIPETTE ENTRY TIMES FOR NEUTROPHILS FOLLOWING STIMULATION WITH 10^{-7}M fMLP

	CONTROL		fMLP STIMULATED	
PIPETTE DIAMETER (μm)	n	MEDIAN ENTRY TIME (s)	n	MEDIAN ENTRY TIME (s)
4.5	40	1.32	15	10.40
			6	>60
6.4	50	0.31	25	5.27
7.4	25	0.14	40	1.32
			2	>60

TABLE 4.2

MICROPIPETTE ENTRY TIMES FOR NEUTROPHILS EXPOSED TO 5 PUFFS OF VAPOUR PHASE CIGARETTE SMOKE *IN VITRO*

	CONTROL		SMOKE EXPOSED	
PIPETTE DIAMETER (μm)	n	MEDIAN ENTRY TIME (s)	n	MEDIAN ENTRY TIME (s)
4.3	50	0.55[a]	66	1.96***
			7	>60
4.7	100	0.47[a]	17	6.08***
			64	>60
5.5	100	0.22	45	0.64***
			12	>60
6.7	100	0.21	20	0.24***
			63	>60

[a] sham exposed neutrophils

comparing smoke exposed with control neutrophils, *** $p<0.001$

TABLE 4.3

MICROPIPETTE ENTRY TIMES FOR NEUTROPHILS EXPOSED TO 1 PUFF OF VAPOUR PHASE CIGARETTE SMOKE *IN VITRO*

		MEDIAN ENTRY TIME (s)		
PIPETTE DIAMETER (μm)	n	CONTROL	n	SMOKE EXPOSED
4.7	60	0.52[a]	64	3.34***
			25	>60
6.0	100	0.40	20	1.0***
			31	>60
6.7	100	0.10	30	0.36***
7.5	50	0.15[a]	20	7.9
			16	>60
8.4	100	0.08	5	3.0***
			20	>60

[a] sham exposed neutrophils

comparing smoke exposed with control neutrophils, *** $p<0.001$.

TABLE 4.4

MICROPIPETTE ENTRY TIMES FOR SMOKE EXPOSED NEUTROPHILS WITH SPHERICAL AND BLEBBED MORPHOLOGY

PIPETTE DIAMETER (μm)	SMOKE DOSE (PUFFS)	MEDIAN ENTRY TIME (s) SPHERICAL MORPHOLOGY	BLEBBED MORPHOLOGY
4.3	5	1.34	4.8
4.7	1	0.53	16.2
5.5	5	0.53	1.57
6.0	1	0.10	1.46
6.7	3	0.11	1.19

TABLE 4.5

THE EFFECT OF CIGARETTE SMOKE CONDENSATE ON NEUTROPHIL DEFORMABILITY AND ELASTASE RELEASE

TREATMENT	n	INCUBATION TIME (min)	MEAN FILTRATION PRESSURES AT 6 MINUTES (cm H_2O)	ELASTASE RELEASE ($ng.ml^{-1}$)	VIABILITY
CONTROL	6	4	3.42 (0.9)	5.1 (3.3)	98%
CONDENSATE (1%)	6		3.9 (2.0)	3.6 (2.2)	98%
CONTROL	5	30	-	31.4 (7.9)	97%
CONDENSATE (10%)	5		-	100 (48.1)*	60-65%

compared with control, * $p<0.05$

TABLE 4.6

REDUCTION OF NITRO BLUE TETRAZOLIUM (NBT) BY NEUTROPHILS AS A MEASURE OF ENDOGENOUS OXIDANT PRODUCTION

	%NEUTROPHILS FORMAZAN STAINING PER CELL			
	-	+	++	+++
CONTROL	67	24	9	0
PMA	6^{+}	16	32	46^{++}
SHAM	40	37	18	5^{*}
SMOKE	35	38	24	3^{*}

Per subject 100 cells were scored as follows: - = less than 5 grains per cell; + = 5 to 10 grains per cell; ++ = 10 to 20 grains per cell; +++ = > 20 grains per cell. Mean values for three subjects are shown.

TABLE 4.7

MORPHOMETRY OF CIGARETTE SMOKE EXPOSED NEUTROPHILS

	MEAN (SD) 200 CELLS	
MEASUREMENT	SHAM EXPOSED	SMOKE EXPOSED
MINIMUM DIAMETER (μm)	6.0 (1.2)	6.1 (1.3)
MAXIMUM DIAMETER (μm)	7.3 (1.9)	8.1 (1.8)***
CIRCUMFERENCE (μm)	24.1 (5.6)	27.4 (5.2)***
AREA (μm^2)	32.4 (11.7)	34.1 (10.8)

compared with sham exposed cells, *** $p<0.001$

4.5 DISCUSSION

4.5.1 THE EFFECTS OF SMOKE EXPOSURE

NEUTROPHIL DEFORMABILITY

The aim of this chapter was to investigate the effect of cigarette smoke exposure on neutrophil deformability and function. *In vitro* exposure to vapour phase cigarette smoke caused a dose dependent decrease in neutrophil deformability as measured during constant flow filtration (Figure 4.4). However, assessment of individual cell deformability, by measuring transit time of neutrophils through 8 μm pores using the Cell Transit Analyser (CTA), showed no difference for sham and smoke exposed neutrophils (Figure 4.5a). The discrepant data obtained for the two filtration systems for the effect of smoke exposure on neutrophil deformability may be due to the different pore sizes for the membranes employed in each system. Membranes with 5 μm pores were used in the constant flow filtration system, whereas 8 μm pore membranes were used in the CTA. The influence of pore size on neutrophil retention in micropore filters has been demonstrated by others (Downey, 1988; Nash, 1988b), who found a decreased pore retention for neutrophils as the pore size of the filters increased. Pore transit times for sham and smoke exposed neutrophils were also assessed using a 5 μm pore membrane in the CTA, however the long transit times which resulted, even with a greater hydrostatic pressure of 18 cm H_2O, lead to the majority of pulses being rejected. Moessmer and colleagues (1990) likewise found 5 μm pores were to small to allow measurement of neutrophil pore transit and hence deformability using the CTA. Thus, at present, 8 μm pore membranes must be employed to measure neutrophil pore transit times using the CTA.

An increase in pore transit time was, however, measured for fMLP stimulated neutrophils using the CTA and 8 μm pores (Figure 4.5b), in agreement with the reduced filterability observed for the constant flow filtration system (section 2.4.3). Hence, it appears factors other than a difference in membrane pore size influenced the pore transit of smoke exposed neutrophils.

MICROPIPETTE STUDIES

As the CTA studies were not conclusive, individual cell deformability was determined for sham and smoke exposed neutrophils by measuring cell entry times for aspiration into micropipettes. The effect of different pipette diameters on pipette entry time for smoke exposed neutrophils was also examined. Control or sham exposed and smoke exposed neutrophils were aspirated into pipettes ranging 4.3 to 8.4 μm in diameter. As greater deformation was required for entry into the smaller

pipettes, the aspiration pressures were adjusted to allow entry times of approximately 0.5 seconds for control neutrophils with all sizes of pipettes.

Micropipette aspiration of smoke exposed neutrophils revealed that those with a blebbed morphology were markedly less deformable than control neutrophils, with a large proportion of the cells unable to enter the pipettes within a 60 second time limit (Tables 4.2 and 4.3). However, smoke exposed neutrophils with normal round morphology had pipette entry times similar to control cells (Table 4.4). These data suggest a heterogenous response to smoke exposure for neutrophils, although differences in cell size could also be a major contributing factor. The faster median pipette entry times measured for neutrophils for increasing pipette diameters suggests that less cell deformation was required for cell entry into the larger sized pipettes. However, even for the larger pipettes, a proportion of smoke exposed neutrophils would not enter. These cells appeared to be hindered by the presence of membrane blebs (Figure 4.7) which prevented the cell from fitting tightly into the pipette tip. As fluid continued to flow into the pipette, the aspiration pressure was insufficient to aspirate the cells.

The entry of fMLP stimulated neutrophils into the different sizes of micropipettes was also influenced by cell shape. Polarisation and hence elongation of the cells, such that the minimum diameter of the cell was less than that of the pipette, allowed rapid cell entry as minimal deformation was required.

4.5.2 EFFECT OF SMOKE EXPOSURE ON NEUTROPHIL MORPHOLOGY:

CELL SHAPE

The shape change observed for smoke exposed neutrophils was different from the shape of neutrophils activated by triggers such as PMA (Lannan, 1992) or fMLP (Watts, 1991). As mentioned above, smoke exposed neutrophils developed membrane blebs and, although morphometric measurement of smoke exposed neutrophils found an increased maximum diameter (Table 4.7), the pseudopodal extensions characteristic of activated cells were not observed.

Bleb formation or zeinosis is believed to occur as a result of cell injury (Trump, 1984), and has been reported for various cell types following oxidant exposure (Edelhauser, 1976; Hayashi, 1987; Hinshaw, 1988; Hinshaw, 1991; Mirabelli, 1988a, 1988b & 1989; Rasp, 1978). Membrane blebs have also been demonstrated on alveolar macrophages obtained from smokers and from smoke exposed hamsters (Rasp, 1978).

The influence of cell shape observed for micropipette entry may explain the anomalous results obtained for the deformability of smoke exposed neutrophils with the CTA and constant flow filtration system. As found for the micropipette studies, a subpopulation of smoke exposed neutrophils could have been prevented from entering the CTA micropore membrane by the presence of blebs on the plasma membrane of the cell. This would suggest the micropore transit times measured for smoke exposed neutrophils using the CTA reflects only the pore transit times for smoke exposed cells with spherical morphology. Moreover, that the efficiency of pulse collection by the CTA was unchanged for smoke exposed compared with sham exposed neutrophils may be explained by blebbed neutrophils not completely sealing the membrane pores so that the presence of the cells at the pores was not registered by the CTA.

CELL SIZE

Cell size may also influence the pore and capillary transit of neutrophils. A larger cell size could delay neutrophil pulmonary transit by enhancing the existing size discrepancy between neutrophils and the microvasculature, particularly in the upper lung regions where the capillaries are believed to be smaller than those of lower lung regions (Glazier, 1969; West, 1964). This is also the site where centrilobular emphysema predominates in smokers (Thurlbeck, 1963).

Although osmotic swelling of neutrophils was found to impair cell filterability, the changes in cell filtration were not as marked as those following stimulation with fMLP or phorbol esters (Nash, 1988a). Likewise, Moessmer and associates (1990) found the range of neutrophil volumes, measured using an electronic sizing system, was less than half the range of neutrophil transit times measured by a CTA, again suggesting that other factors in addition to volume determines neutrophil transit times through pores. Moreover, Pecsvaraday and colleagues (1992) observed a decrease in cell volume following 60 seconds stimulation with fMLP, despite an increase in cell transit time through 8 μm pores over the same period.

The importance of cell size was also investigated by Doerschuk and co-workers (1990a) using an indicator-dilution procedure in rabbits. Their study showed that leucocyte size correlated with the extraction of the cells, i.e. the retention of neutrophils, on their first transit through the pulmonary capillary bed (Doerschuk, 1990a). Downey and colleagues (1990) replicated this study using an *in vitro* capillary model consisting of a constant flow filtration system with 5 μm pore membranes. The influence of cell size on the transit of cells through these model capillaries was evident from the ease with which platelets passed through relative to

the larger leucocytes. Also, the smallest of the leucocytes, the lymphocytes, had less retention in the filter (Downey, 1990) and *in vivo* in the microvasculature (Doerschuk, 1990a), compared with the larger neutrophils and monocytes.

Smith and colleagues (1986) observed the deformability of pulmonary alveolar macrophages obtained from smokers and smoke exposed hamsters. Although macrophages obtained from hamsters that had inhaled filtered, i.e. vapour phase cigarette smoke had normal filterability, exposure to whole particulate cigarette smoke reduced the filterability of human and hamster macrophages. This impaired filterability was associated with an increase in macrophage size and hence a reduction in the excess surface area available for cell deformation. However, the markedly impaired filtration of smoke exposed neutrophils through micropore membranes was not due to an increase in cell size as the area of the cell (measured from TEM's) was unaltered (Table 4.7).

4.5.3 FUNCTIONAL ACTIVITY OF SMOKE EXPOSED NEUTROPHILS

An increase in the maximum diameter of the cell with a change in cell shape has also been noted following cell activation (Keller, 1990). Shape change is considered to be a sensitive indicator of cell activation (Haslett, Guthrie et al., 1985). Moreover as demonstrated in this thesis (chapter 2) and by others (Frank, 1990a; Nash, 1988a; Pecsvarady, 1992; Worthen, 1989), activated neutrophils have a reduced filterability, reflecting a reduction in cell deformability. In addition, *in vivo* studies show enhanced retention of fMLP (Peters, 1992; Worthen, 1989) and C_{5a} (Lien, 1991) stimulated neutrophils due to increased cell stiffness. Hence, the reduced deformability observed for *in vitro* smoke exposed neutrophils suggested that the cells may be activated.

However, in this study there was no functional evidence of neutrophil activation by *in vitro* smoke exposure. Neither exogenous (Table 4.6) or endogenous oxidant production (Figures 4.9a and b) from neutrophils, nor elastase release (section 4.4.5) or functional proteolytic activity (Figure 4.10) were enhanced by *in vitro* smoke exposure. Likewise, Corberand and associates (1980) reported a decreased NBT reduction for neutrophils following incubation with water-soluble extracts of cigarette smoke. Also, Eichel and Shahrik (1969) found a depressed metabolic function for leucocytes obtained from the mouth following one cigarette. More recently, suppressed oxidative metabolism was reported by Beswick and colleagues (1986) in peripheral blood neutrophils from smokers.

In contrast, enhanced radical production and elastase release, indicating neutrophil activation, as a result of *in vivo* or *in vitro* smoke exposure has been reported (Abboud, 1986; Anderson, 1974; Corberand, 1979; Gillespie, 1987; Hind, 1991; Hoidal, 1981; Ludwig, 1982; MacNee, 1989a; Richards, 1989; Weitz, 1987). Moreover, Anderson and colleagues (1991) observed priming of neutrophil chemotaxis and radical release by 3 hours of passive smoking. Furthermore, Hickey and co-workers (1993) found those neutrophils that were sequestered in the microvasculature of rat lungs were primed for elastase release by smoke exposure. Also, Bosken and associates (1991) reported a transient increase in the levels of plasma myeloperoxidase, a neutrophil enzyme which is thought to cause tissue damage by mediating hydrogen peroxide oxidation of halides to produce the oxidant hypochlorous acid (Weiss, 1989), during acute smoking.

Several factors may explain these contrasting observations. Admittedly, in the present study the neutrophils were exposed to a relatively high dose of cigarette smoke without the barrier of the alveolar capillary membrane barrier which is present *in vivo*. However, the actual dose and components of cigarette smoke to which neutrophils may be exposed, while in transit in the pulmonary capillaries, is not known. Doerschuk and colleagues (1988b) observed a peak increase in the COHb levels in the blood of rabbits following inhalation of 1 puff of cigarette smoke when sampled directly from the aorta, which was much higher than the COHb measured in the peripheral arterial or venous blood. However, the amount smoked *in vivo*, and the time interval between the last cigarette and blood sampling could also influence the results. Moreover, the *in vivo* studies would be subject to individual variations between the blood donors, more so than the *in vitro* studies. Furthermore, neutrophils *in vivo* may, after the direct effect of smoke, receive stimulation for example from cytokines released by smoke-activated alveolar macrophages and endothelial cells, resulting in cell activation. Also the isolated neutrophils used for *in vitro* studies may be altered by the harvesting procedure.

4.5.4 RECOVERY OF NEUTROPHIL DEFORMABILITY AND FUNCTION

The injury induced by *in vitro* smoke exposure was reversible. Neutrophil deformability improved following incubation for 1 hour in fresh PBS; PBS containing the antioxidant glutathione; and, most effectively, following incubation in autologous plasma (Figure 4.12). Moreover, spontaneous O_2^- release and the stimulated H_2O_2 and O_2^- release from neutrophils incubated for one hour in plasma (Figure 4.13) were significantly greater than the levels measured immediately following smoke

exposure (Figure 4.9), although a full recovery was not observed within one hour. Bridges and colleagues (1977) assessed the reversal of the inhibition of neutrophil chemotaxis induced by incubation for 15 minutes with water soluble fractions of whole cigarette smoke. Removal of the smoke fractions slightly reversed the inhibition of neutrophil chemotaxis. However, although the authors found that 10 mM cysteine completely protect against the effect of soluble smoke fractions when present during incubation, the later addition of cysteine did not improve neutrophil chemotaxis. As the chemotaxis assay was performed over 2 hours, the lack of recovery was unlikely to be due to an inadequate incubation period with cysteine. It is possible that the active motility required for chemotaxis is more sensitive to the effects of smoke than passive deformation. However, the difference in the results may simply be a reflection of the methods of smoke exposure and the longer exposure time used by Bridges et al (1977).

4.5.5 THE REDUCED FILTERABILITY IS NOT DUE TO INCREASED NEUTROPHIL ADHESIVITY FOLLOWING SMOKE EXPOSURE

Neutrophil retention in micropore membranes following *in vitro* smoke exposure, and in the lung microvasculature during smoking *in vivo* could also be due to increased adhesivity. The monoclonal antibody (CD18Mab) which blocks CD18, a major neutrophil surface adhesion glycoprotein (Albeda, 1991), has been shown to block the adhesion of activated neutrophils to endothelial cells (Vedder, 1988a; Zimmerman, 1988), epithelial cells and plastic (Selby, 1992). Hence, to determine whether the reduced filterability observed with smoke exposed neutrophils was due to increased adhesion to the filter, neutrophils were preincubated with CD18Mab.

The filtration of smoke exposed neutrophils through micropore filters was, however, unaffected by pre-incubation with CD18Mab. Likewise, as found by other workers (Downey, 1990; Worthen, 1989), pre-incubation with CD18Mab did not affect the filtration pressures developed by stimulated neutrophils. Hence, neutrophil adhesivity, at least that which is mediated via CD11/CD18, does not influence retention in micropore filters. Indeed functional adhesion of smoke exposed neutrophils to plastic or to epithelial cells was reduced following *in vitro* smoke exposure (Selby, 1992). Also acute smoking *in vivo* of 4 cigarettes did not alter CD18 expression on peripheral blood neutrophils (Selby, 1992). Moreover, although fibronectin degradation by smoke exposed neutrophils was not significantly depressed a reduction occurred in most subjects. Fibronectin degradation is dependent on neutrophil adhesion to the matrix (Campbell, 1987). In contrast, *in*

vivo increased leucocyte rolling and adhesion to arterioles and post-capillary venules was observed in the dorsal skin fold of smoke exposed hamsters (Lehr, 1993). Although a stimulant-induced increase in adhesion in the systemic circulation is not always matched by increased adhesion in the pulmonary circulation (Doerschuk, 1990), Klut and associates (1993) found an increase in the expression of CD18 following smoke exposure on the surface of neutrophils sequestered in the microvasculature of rabbit lungs. The dichotomy between *in vivo* and *in vitro* effects of smoke may again relate to the presence of a second stimulus *in vivo* which is not present *in vitro*.

Although there are only few studies on neutrophil adhesion and smoke exposure, there are several reports on the effect of smoke on macrophage adhesivity. Rasp and colleagues (1978) found alveolar macrophage obtained from smokers were less adherent to nylon fibre than non-smokers which may explain the increased numbers of alveolar macrophage obtained routinely by bronchoalveolar lavage fluid from smokers. Similarly, reduced adherence was reported for rabbit alveolar macrophage following *in vivo* smoke exposure (Green, 1967) or incubation with smoke extracts *in vitro* (Low, 1977). However, conflicting reports have also been published. In two studies smoker's alveolar macrophage were more adherent to plastic culture dishes (Laviolette, 1981) and glass cover slips (Mann, 1971) than non-smoker's macrophages. However, the latter group had pre-selected their population by studying only those cells that had previously adhered to glass during a 4 hour incubation period. Moreover, in both studies bacteria or zymosan was present during the incubation period which may have activated macrophage phagocytosis. The increased adherence observed in these studies may thus be due to activation of the cells. Enhanced expression of leucocyte adhesion molecules (CD11/CD18) and functional adherence to cultured umbilical vein endothelial cells was shown by Schaberg and colleagues (1992) to be higher for smoker's than non-smoker's alveolar macrophages. Moreover, Pittilo and associates (1982 & 1990) noted aggregation and increased adhesion of platelets to aortic endothelium following acute and chronic smoke exposure in the rat. The same authors also found platelets in blood sampled from non-smokers who smoked 2 cigarettes adhered to rabbit aortic endothelium which was not observed with platelets from the pre-smoking blood sample. However, no smoke exposed neutrophils were found adhered to the rabbit endothelium which suggests no increase in neutrophil adhesivity following smoke exposure, although this may be due to the species difference (Pittilo, 1984).

The formation of neutrophil aggregates, produced by an increase in cell-cell adhesivity, could also be the cause of delayed pulmonary transit of neutrophils *in*

vivo and increased retention in the micropore membrane *in vitro*. Enhanced leucocyte aggregability has been reported as a result of smoking (Ricevuti, 1990), however cell-cell adhesion following smoke exposure *in vitro* was not apparent under light microscopy in the present studies.

Thus, there is evidence for both an increase and a decrease in the adhesion of leucocytes as a result of *in vitro* or *in vivo* smoke exposure. However, as the technique used for smoke exposure in this thesis depressed neutrophil adhesion, and preincubation with CD18Mab did not alter their filterability, the impaired filterability of smoke exposed neutrophils in these studies appears to reflect a decrease in cell deformability and does not seem to be due to increased adhesion to the micropore filter.

4.5.6 COMPONENTS OF CIGARETTE SMOKE

Evaluating the biological effects of cigarette smoke is hindered by its complex composition. Smoke which is inhaled and retained in the lungs is different than the smoke which comes directly from the cigarette (Pryor, 1982). Inhalation through the mouth absorbed 60% of the water soluble, and 25% of the insoluble components of smoke (Dalhamn, 1968). However, the retention of compounds such as acetaldehyde, isoprene, acetone, acetonitrile, toluene, carbon monoxide, and particle matter occurred mainly in the lungs (Dalhamn, 1968).

It is not clear which components of cigarette smoke penetrate the alveolar membrane into the blood stream. Nicotine and its major metabolite cotinine, thiocyanate, and carbon monoxide have been measured in serum and/or urine of smokers to quantitate cigarette smoke intake (Barlow, 1987; Benowitz, 1989) which demonstrates the penetration of such components into the blood stream. Likewise, Buckley and associates (1975) suggested penetration of ozone through the epithelial/endothelial barrier of the lungs by observing evidence of oxidant stress in blood following acute ozone exposure. Also nitric oxide, another major constituent of smoke, was found to pass rapidly into the blood (Yoshida, 1980).

NICOTINE

Several investigators have investigated the effect of isolated smoke components on the locomotory and metabolic function of neutrophils (Bridges, 1977; Corberand, 1980; Eichel, 1969). In the present study the effect of nicotine on neutrophil deformability was investigated. Plasma nicotine concentration have been shown to

correlate with the increased white cell count observed in smokers (Taylor, 1986b). Also, nicotine is known to be chemotactic for neutrophils (Totti, 1984) and may therefore activate cells. However, no change in deformability was evident which, even at the lowest dose used, was 10 fold higher than the levels reported for smokers (Russell, 1976; Taylor, 1986b). Likewise, Corberand and colleagues (1980) found neutrophil metabolic and chemotactic activity was unaltered by 30 minutes incubation with nicotine concentrations equivalent to those used in this study, although random migration was affected at the highest concentration. Also nicotine did not affect neutrophil superoxide production or degranulation at similar concentrations (Totti, 1984).

CIGARETTE SMOKE CONDENSATE

Water-soluble components or whole-unfractionated smoke condensates in an organic solvent such as DMSO are frequently used to investigate the effects of smoke exposure *in vitro*. Blue and Janoff (1978) calculated that the physiological concentration of smoke components in the lung surfactant layer is approximately 1 $mg.ml^{-1}$ for a person smoking 20 cigarettes per day, presuming retention of only 1% of the total condensate burden. Blue and Janoff (1978) found that unfractionated cigarette smoke condensate could induce elastase release from human neutrophils, which supported the hypothesis that neutrophils were central to the pathogenesis of smoking-induced emphysema. Neutrophil deformability and elastase release were assessed following incubation in the tonometer with cigarette smoke condensate as a comparison to fresh cigarette smoke exposure.

Exposure to cigarette smoke condensates did not affect neutrophil deformability at concentrations which were within the 'physiological' range where cell viability was maintained (Table 4.5) Likewise, 4 minutes exposure to 1% did not induce elastase release(section 4.4.5). However, incubation with 10% condensate for 30 minutes, which was equivalent to the 1% condensate used by Blue and Janoff (1978), resulted in increased elastase release but a loss of cell viability was also observed. That cigarette smoke condensate had no effect on neutrophil deformability, in contrast to the marked effect observed following exposure to vapour phase cigarette smoke, may be due to the solvent DMSO which has radical scavenging properties (Halliwell, 1989). Moreover, the gas-phase radicals are thought to adsorb onto particulate components of smoke, 'quenching' the reactive oxygen radicals, and hence some of the toxicity of whole smoke and whole-smoke condensates (Borland, 1985).

In summary, the studies in this chapter have shown that neutrophils exposed to vapour phase cigarette smoke *in vitro* had a reduced ability to filter through a micropore membrane, the dimensions of which are comparable to the average dimensions of the human pulmonary capillary segments. The decrease in cell filterability was not caused by CD18-mediated neutrophil adhesion to the micropore membrane, nor was it associated with an increase in cell size as a result of cigarette smoke exposure, but a change in cell shape was evident. However, the altered cell shape and reduced deformability was not associated with functional activation of the cells as determined by the spontaneous release of reactive oxygen intermediates and proteolytic activity. Both cell deformability and function showed signs of recovery after 1 hour in plasma.

The impaired filterability found for *in vitro* smoke exposed neutrophils may explain the enhanced retention of neutrophils in the lungs observed during smoking (Bosken, 1991; MacNee, 1989d).

CHAPTER 5
A MECHANISM FOR THE SMOKE-INDUCED CHANGE IN NEUTROPHIL DEFORMABILITY AND FUNCTION

5.1 INTRODUCTION

In the previous chapter *in vitro* cigarette smoke exposure had a detrimental effect on neutrophil deformability and function. The smoke exposed cells were, however, able to recover their deformability and functional activity with time when incubated with plasma. These data suggest the transient enhanced lung retention of neutrophils during acute cigarette smoking (Bosken, 1991; MacNee, 1989d) may be due to a temporary reduction in cell deformability. The aim of this chapter was to determine the mechanism of the smoke-induced reduction in neutrophil deformability.

A reduction in neutrophil deformability occurs following cell activation (Frank, 1990a; Moessmer, 1990; Nash, 1988a; Neumann, 1990), which is associated with changes in the cytoskeletal network of the cell (Howard, 1984 & 1985; Packman, 1990; Wallace, 1984). Remodelling of the cell cytoskeleton is intrinsic to many of the neutrophil's functions such as chemotaxis, secretion and phagocytosis. Three types of cytoplasmic fibres have been identified and characterised as the main constituents of this network: the actin filaments, microtubules and intermediate filaments.

5.1.1 THE CELL CYTOSKELETON

ACTIN

Actin is one of the most abundant proteins in the neutrophil and contributes to both structural and functional cellular activity (Howard, 1984; Roos, 1987; Stossel, 1989). Actin exists in two conformations; the monomeric globular form (G-actin), and the filamentous form (F-actin) which is a polymer of 7-12 nm diameter comprised of actin monomers (Janmey, 1990). Actin filaments are composed of two strands of G-actin twisted into a helix. Actin monomers are bound by calcium and a non-covalently bound adenine nucleotide (either ADP or ATP) for stability against denaturation (Asakura, 1961). Polymerisation of these subunits occurs when the monomer is activated and nucleation (trimer formation) initiated (Carson, 1986). Rapid addition of monomers then results in the formation of an actin filament. If the polymer is ATP bound, a stiff filament results due to hydrolysis of the terminal phosphate group of ATP, whereas a filament consisting of ADP-monomers is flexible (Janmey, 1990). In the resting cell, 30% of actin is in the filamentous form which increases to about 60% following stimulation (Cassimeris, 1990).

Although G-actin addition and dissociation occurs at both ends of the filament, the filament is a polar structure with growth predominantly at the "barbed end" and dissociation predominantly at the "pointed end". The incorporation of a monomer

molecule onto the filament releases a terminal phosphate from the bound ATP, but filament growth is non-energy dependent (Asakura, 1961). Under normal conditions the rate of addition and dissociation is constant, termed treadmilling, and thus the net rate of polymer growth is zero (Neuhaus, 1983). Following stimulation, monomer addition is faster than dissociation, resulting in polymer elongation (Cano, 1991)

Actin filaments in neutrophils can form in the cytoplasm or as protrusions from the cell. Within the cytoplasm, actin filaments can produce a thin 3-dimensional network important for mechanical support of the cell (Pollard, 1984). The actin filaments that protrude from the cell were first described as 'fibrous projections' by Porter, Claude and Fullham (1945) in 1945 and associated with cell migration. Since then several different forms of cytoskeletal protrusions have been identified.
Microspikes are stress fibres which protrude out of the leading edge of the cell and exert a contractile force by attachment to adhesion plaques (Burridge, 1987; Malech, 1977). Lamellipodia or pseudopods are large flattened sheets composed of actin and devoid of organelles, which are orientated and 'lead' in the direction of locomotion (Coates, 1992; Malech, 1977; Oster, 1987). These pseudopods either form a permanent attachment to substratum or are swept back as "ruffles" in a wavelike motion. The anchored tail end of the migrating neutrophil is also composed of a small bundle of actin filaments, termed a uripod. Another protrusion, observed predominantly in injured cells, are membrane blebs (Oster, 1987).

MICROTUBULES

Microtubules are similar to actin filaments in that they are composed of subunits of α and β tubulin which assemble into a polarised, helical polymer by a similar nucleation and elongation process as for actin, with growth faster at one end than the other (Kristofferson, 1986). This polymer is the largest of the cytoskeleton (25 nm diameter). Hydrolysis of a phosphate group also occurs, but, in this case, from a guanine nucleotide (GTP)(Olmsted, 1973). The microtubules' primary function is to organise the internal contents of neutrophils including intracellular transport of material (Olmsted, 1973). Microtubules are also involved in the maintenance of cell form and cellular motility. By forming the inner core of pseudopodal extensions, which are linked to the internal portion of the cell, microtubules allow the cell to extend without adhesion to substratum (Malech, 1977; Olmsted, 1973).

INTERMEDIATE FILAMENTS

Intermediate fibres are 10 nm in diameter and thought to be composed of three or four strands of subunits, with specific protein subunit for different cell types. The subunits differ from one another in the size and amino acid sequences of their N-terminal and C-terminal domains. However, each subunits contains a central helical rod domain of conserved structure that forms the core of all intermediate fibres (Steinert, 1985). Few data are available to define the function of intermediate filaments, although several roles such as cell shape definition, movement of organelles and mechanical coordination of the cytoskeleton have been suggested (Alberts, 1983; Steinert, 1984).

THE MICROFILAMENT NETWORK

The physical stability and mechanical behaviour of the microfilament network is dependent on connections between the fibrous elements. Each fibre has its own specific crosslinking proteins present in the cytoplasm. For actin, three types of actin-binding proteins exist: the monomer sequestration proteins; the capping proteins; and the crosslinking proteins.

Monomer sequestration proteins, such as profilin and thymosin β4 and β10, bind to actin monomers, decreasing the polymerisable pool of actin monomers (Lassing, 1985; Yu, 1993). Thymosin β4 is present in cells at high concentration and therefore maintains much of the actin in the unpolymerised state. It is not clear how actin is released from thymosin β4 during cell activation. Profilin, as a monomer binding protein, was also though to inhibit actin polymerisation. However, as profilin-actin binding is inhibited by profilin's association with phosphatidylinositol 1,4 bisphosphate (PIP_2)(Lassing, 1985), the increased affinity of phospholipase C for PIP_2 during cell activation allows dissociation of profilin from the cell membrane, which is unexpected at a time when actin polymerisation takes place. However, experiments have shown that profilin catalyses the exchange of ADP for ATP on bound actin monomers (Mockrin, 1980). ATP-actin has a lower critical concentration (the monomer concentration at which filaments are stable) than ADP-actin (Pollard, 1986), thus profilin prepares actin monomers for more rapid actin polymerisation during cell activation.

Several capping proteins, such as gelsolin, gCAP, Cap Z and aginactin control the addition and loss of subunits by binding to the barbed end, thereby controlling actin polymerisation (Bearer, 1993). Moreover, if agonists induce the filament to become uncapped, rapid polymerisation could result (Bearer, 1993). Capping proteins also allow filaments to be bound to other structures, such as adhesion plaques in

fibroblasts and desmosomes in epithelial cells. Capping proteins can be divided into two main groups, those that are calcium-dependent, some of which can also sever actin filaments, and the calcium-independent capping proteins (Bearer, 1993). Gelsolin was observed to sever filaments by binding to the sides of actin filaments (Yin, 1979). The severing of filaments increases the sites for monomer addition, and thus overall actin polymerisation in the cell, but also allows increased motility by severing stress fibres with consequential loss of firm adhesion.

Crosslinking proteins bind existing actin filaments to form the 3-dimensional structure of the cytoskeleton. For example, villin links actin filaments into bundles; α-actinin and filamin allow linking in any direction; and fibrin mediates parallel crosslinking (Alberts, 1983; Bearer, 1993; Pollard, 1984) The amount of crosslinking determines the liquid or sol behaviour of the network. Gelation of the actin network increases the elastic rigidity and viscosity of the cell (Sato, 1985), thereby affecting the cell's resistance to deformation (Elson, 1988; Worthen, 1989).

METHODS FOR STUDYING ACTIN POLYMERISATION

Two classes of drugs, the cytochalasins and phallotoxins, have been valuable in ascertaining the mechanism of actin polymerisation (Sampath, 1991)(Cassimeris, 1990). For several years it has been widely accepted that cytochalasins bind with high affinity to the barbed ends of actin filaments, thereby inhibiting the addition and loss of actin monomers (Brown, 1979). However, more recent studies have demonstrated that inhibition of actin filament elongation cannot be explained by barbed-end capping alone. Sampath and Pollard (1991) demonstrated that both cytochalasin B and D altered the rate of elongation at both ends of the filament but inhibition was not complete with slow growth occurring at both ends (Bonder, 1986). Also, at high concentrations, cytochalasin D was found to bind to actin monomers (Goddette, 1986).

Phallotoxins, a family of poisonous cyclic peptides derived from the poisonous mushroom *Amanita phalloides*, and are highly specific for filamentous actin (Barak, , 1980; Wulf, 1979). They are reported to stabilise actin filaments by inhibiting monomer dissociation from both ends (Sampath, 1991). Moreover, subunit addition to the barbed end of the filament was inhibited by about 50% by the phallotoxin phalloidin (Colluccio, 1984). Sampath and Pollard (1991) proposed a stronger interaction between actin subunits and phalloidin than between the subunits themselves, thereby creating unfavourable circumstances for free monomer binding, as a mechanism of action for phalloidin. Phallotoxins have a low molecular weight

(phalloidin 789, phallacidin 848) allowing entry into living cells after minimal membrane permeabilisation,

Many workers have demonstrated actin filament generation in activated cells by disruption of the F-actin assembly and organisation using cytochalasins, or by specific labelling of the actin filament. Fixed cells can be fluorescently stained by fluorescent heavy meromyosin (Sanger, 1975); microinjection of fluorescent actin (Taylor, 1978); or indirectly with anti-actin antibodies (Lazarides, 1974). However, phallotoxins are now used predominantly. Barak and co-workers (1980) developed phalloidin as a fluorescent marker for F-actin by coupling 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD), a fluorophore of small size (200 mw), to the carboxylic acid residue of the toxin.

ACTIN POLYMERISATION AND CELL DEFORMABILITY

Studies using actin inhibitors or fluorescence staining of actin filaments demonstrate the contribution which the mechanical properties of the neutrophil make in cell retention in micropore filters *in vitro* (Erzurum, 1991; Frank, 1990a; Worthen, 1989) and in capillaries *in vivo* (Doerschuk, 1989; Worthen, 1989). Worthen and colleagues (1989) demonstrated the role of actin filaments in fMLP-induced neutrophil stiffness by abolishing micropore retention of activated cells by the addition of cytochalasin D. Moreover, they established, using cytochalasin D, that the retention of fMLP stimulated neutrophils *in vivo* in the rabbit was due to actin-induced cell stiffness (Worthen, 1987a). Similarly, by staining F-actin using the fluorescent derivative NBD phallacidin, it was found that an increase in actin polymerisation in fMLP-stimulated neutrophils was closely followed by a reduction in deformability (Frank, 1990a) and preceded a change in cell shape (Coates, 1992; Howard, 1985).

The importance of the cell's cytoskeletal assembly in the retention of neutrophils in capillary-sized pores was also shown in studies using the promyelocyte HL60 cell line (Erzurum, 1991). HL60 cells can be induced to differentiate towards granulocytes by addition of DMSO. With differentiation the cell volume is reduced and the F-actin organisation changes from a thick cortical rim with focal areas, to a thin rim. Moreover, differentiated HL60 cells can respond to fMLP by reorganising their F-actin in manner similar to neutrophils (Erzurum, 1991). However, although these cells express adherence glycoproteins on their surface (Hickstein, 1987), their adhesion to serum coated plastic or albumin coated beads was not enhanced by fMLP stimulation (Erzurum, 1991). Thus HL60 cells can be used to investigate the

importance of microfilament organisation in relation to cell deformability in the absence of cell surface adhesive properties.
Erzerum and colleagues (1991) found HL60's which were differentiated into granulocytes were more deformable than undifferentiated cells. Disruption of the cell microfilament organisation with cytochalasin D increased the deformability of undifferentiated cells. Similarly, the reduced deformability of fMLP stimulated differentiated cells was increased by addition of cytochalasin D (Erzurum, 1991).

5.1.2 THE CELL MEMBRANE

Mammalian cell membranes consist of a bilayer of lipids which are generally amphipathic molecules i.e. the hydrocarbon regions have little affinity for water and stay together, as do the polar hydrophilic parts. Animal cell membranes are composed primarily of phospholipids (phosphoglycerides and sphingomyelin) and some membranes, particularly plasma membranes, contain hydrophobic sterols (cholesterol) (Bleich, 1977; Singer, 1974). Proteins that are involved in fundamental cellular functions, such as receptor-ligand interaction, carrier-mediated transport, and enzyme activity, are embedded in this lipid bilayer (Singer, 1974).

PLASMA MEMBRANE FLUIDITY

Many of the functions of the neutrophil such as locomotion, phagocytosis (Donner, 1989), protease and radical release are influenced by membrane fluidity (Yuli, 1982). Hence for optimum membrane function the mobility of the lipid bilayer, referred to as membrane fluidity, is required to be in a fluid state. Membrane perturbations that alter the fluidity may affect the metabolic and motile function of the cell by the degree of exposure of receptor proteins, receptor/ligand interaction, trans-membrane transport and the activity of membrane bound enzymes (Chan, 1991; Sinha, 1977; Stubbs, 1983; Yuli, 1982). Moreover, it is known that dietary variations, cell proliferation, cell cycle, ageing, agents such as hormones, drugs and toxins, and pathological states such as neoplastic change can all affect membrane fluidity (reviewed by Aloia (1983)).

The membrane is an ordered fluid, where orientation of lipids and proteins perpendicular to the membrane plane is more probable than into the plane. Fluidity is influenced by the cholesterol - phospholipid ratio; the number of unsaturated double bonds within phospholipid acyl chains; by the class of phospholipid; and the

presence of amphipathic substances which are soluble in both water and lipid (Bleich, 1977; Stubbs, 1984).

The interaction between cholesterol and phospholipids imposes a degree of immobility on the portions of the lipid molecules that are nearest the surface, by increasing the efficiency of packing of phospholipids (where packing is the number of molecules per unit area), resulting in a decrease in the fluidity of the membrane (Cooper, 1975). As sterols are insoluble in water, but solubilised by polar lipids such as the phospholipids, the insertion of cholesterol is dependent on the relative amount of phospholipid in the membrane. Hence, the transfer of cholesterol from plasma lipoproteins to cell membranes is dependent, not on the cholesterol concentration of serum, but the cholesterol-phospholipid ratio (Bleich, 1977).

The relative presence of saturated or unsaturated acyl chains is probably the most common determinant of membrane fluidity. The kink in the acyl chain, induced by the presence of a double bond (unsaturated) which prevents rotation of the groups attached to the carbon atom, does not allow packing of phospholipids as tightly as for saturated (no double bonds) acyl chains. Unsaturated acyl chains, therefore, form disordered membranes with high fluidity, and saturated chains form highly ordered membranes with low fluidity (Patel, 1986).

Furthermore, the relative amounts of the choline-containing phospholipids, lecithin and sphingomyelin, which are primarily located in the outer layer of the membrane, can also determine the fluidity of the membrane (Cooper, 1977). Artificial membranes composed entirely of sphingomyelin are less fluid than membranes consisting only of lecithin despite a similar degree of acyl chain saturation (Cooper, 1977; Schinitzky, 1974)

Amphipathic molecules (soaps, alcohols, detergents and local anaesthetics) partition between the aqueous and lipid phases of the membrane, thereby increasing the fluidity of the phospholipids. Yuli and colleagues (1982) showed that aliphatic alcohols altered the fluidity of the neutrophil membrane which increased the affinity of the fMLP receptor, enhanced chemotaxis, but reduced the cells generation of O_2^- and lysosomal enzyme release.

By changing the physical state and composition of the lipids, the fluidity of the membrane can be altered, which may be necessary to maintain cell function. For example, to maintain the fluidity of the plasma membrane under decreased temperatures either the membrane-lipid composition can be altered in favour of more lecithin, the cholesterol-phospholipid ratio can be decreased, or the degree of acyl chain saturation can be reduced (Hazel, 1974 &1991).

MEASUREMENT OF MEMBRANE FLUIDITY

Membrane fluidity is a widely used concept in membrane research, but the term lacks a precise definition. This is due to the fact that the membrane differs from an ordinary isotropic fluid such as oil, where the motion of the particles in the fluid can be described with one physical parameter, the fluidity. A membrane, on the other hand, is an anisotropic, two-dimensional fluid and the lateral and rotational mobilities of proteins and lipids can not be expressed in terms of one parameter only. Rather a set of physical parameters is required (the lateral and rotational diffusion). A number of techniques have been developed to measure the physical parameters of the membrane which allow quantification of the fluidity of membrane components. These techniques include electron spin resonance spectroscopy (ESR); nuclear magnetic resonance spectroscopy (NMR); Raman spectroscopy; and fluorescence spectroscopy.

ESR and NMR examine the rotational motion of acyl chains. ESR utilises stable free radicals, often described as 'spin-labels', in a magnetic field and the absorption spectrum for microwave radiation derived from such probes allows calculation of the order parameter, i.e. the molecular packing of the membrane lipids. An increase in lipid order is associated with a decrease in membrane fluidity. Analysis of proton, carbon or phosphorous NMR details the movement of atoms in the membrane to indicate the chemical and physical properties of the lipid bilayer. Raman spectroscopy yields basic structural information by measuring scattered light from an incident beam, the intensity of which is a function of its frequency. The frequency difference between the incident light and scattered light corresponds to vibrational frequencies of molecules in the cell such that different bonds within lipids can be distinguished, and the motion around these bonds determined (Andersen, 1978; Stubbs, 1983). Fluorescence is the phenomenon that certain molecules emit light with longer wavelength than the light with which they were illuminated. Such molecules are called fluorophores. Fluorescence spectroscopy utilises fluorophores that are partitioned into specific membrane lipid domains, reflecting either the lateral diffusion of membrane lipids (fluorescence recovery after photobleaching) or the rotational behaviour of lipid acyl chains (rotational relaxation time and fluorescence anisotropy). The principle of fluorescence spectroscopy is that polarisation of light emitted by a fluorescent probe depends on its rotational movement which is a function of the viscosity or fluidity of the domain in which the probe is inserted. Fluorescence emitted by molecules that rotate very rapidly and without restriction will be completely depolarised, whereas fluorophores that are strongly hindered will emit fluorescence with a marked polarisation. The techniques for measurement of

membrane fluidity are described in more detail in reviews by Anderson (1978) and Stubbs (1983).

Fluorescence Recovery After Photobleaching (FRAP) was the technique employed for the present studies which gives a measure of the lateral diffusion of membrane lipids (Axelrod, 1976; Koppel, 1976). In this technique a cell is labelled by insertion of fluorescent lipid or protein probes into the plasma membrane, or saturation of a membrane-localised receptor with the fluorescently-tagged ligand. A small region containing the mobile probes is then subjected to a brief intense exposure of a focused laser beam which causes irreversible bleaching of the fluorophore in that region. The re-appearance of fluorescence within the bleached area results from diffusion of unbleached fluorophores into that area. The fluorescence photobleaching recovery kinetics can be analysed to determine lateral mobility rates (the diffusion coefficient, D_L) and to determine the fraction of the total fluorophore which is immotile (percentage recovery)(Axelrod, 1976). The essentials of this method were first described by Poo and Cone (1974) using rhodopsin in the absence of laser technology.

5.1.3 OXIDANT STRESS

Biological systems are continuously challenged by oxidants that are generated either endogenously (e.g. by phagocytes) or exogenously (e.g. by exposure to cigarette smoke). The tissues of the body are protected from such oxidative challenges by enzymatic and non-enzymatic antioxidants. Oxidative stress is, therefore, thought to occur when there is an imbalance in favour of oxidants, and to be involved in the pathophysiology of ageing and of several diseases (Halliwell, 1987). The lungs are particularly vulnerable to the effects of inhaled oxidants (high doses of pure oxygen, ozone, nitrogen oxide, nitrogen dioxide), or particles (quartz, asbestos, silica) which induce toxic processes.

Ozone and nitrogen dioxide, both components of cigarette smoke, are potent oxidants capable of direct cell damage by inducing lipid peroxidation or generating damaging free radicals (Borland, 1985; Mustafa, 1990; Patel, 1988). These oxidant gases also cause leucocyte accumulation in the lungs resulting in further lung injury by the release of free radicals and proteases from activated blood and tissue phagocytes during an inflammatory reaction (Mustafa, 1978; Schelegle,1991).

THE EFFECT OF OXIDANTS ON THE CELL CYTOSKELETON

Oxidant injury has been associated with changes in the shape and function of a variety of cell types, thought to result from changes in the cell cytoskeleton. Shasby et al (1985) reported that endothelial monolayers exposed to chemically generated oxidants (xanthine/xanthine oxidase) changed shape and retract from adjacent cells, allowing the transfer of macromolecules such as albumin across the monolayer. They demonstrated the cell shape change was associated with changes in the normal pattern of actin filaments. Mirabelli and co-workers (1988a) observed the appearance of numerous membrane blebs when rat hepatocytes were exposed to the thiol oxidant diamide or the redox-cycling quinone menadione, which they proposed was linked to the oxidation of critical sulfhydryl groups in cytoskeletal proteins, resulting in the dissociation of the plasma membrane from the underlying cytoskeleton. In a further study of human platelets, the same group confirmed their hypothesis by demonstrating a reduction in intracellular GSH and protein bound thiols following menadione exposure (Mirabelli, 1989), which was also found for other cell types (Mirabelli, 1988b).

Thiols are a major source of protection against reactive oxygen species. Sulfhydryl (thiol or SH groups)-dependent proteins are involved in many cell functions, such as transmembrane signalling and motility, as these proteins are found in membrane receptors (Nath, 1976) and associated with the cytoskeletal elements involved in cell locomotion (Noble, 1975). Moreover, monomeric actin has four thiol groups, three of which are masked by ATP (Faulstich, 1984). Depletion of ATP, which occurs during oxidative stress (Hinshaw, 1989; Spragg, 1985), can therefore unmask the thiols in actin, increasing their susceptibility to oxidant damage.

THE EFFECT OF OXIDANTS ON THE PLASMA MEMBRANE: LIPID PEROXIDATION

Membrane phospholipids contain a number of unsaturated and polyunsaturated fatty acid side chains. The fatty acid side chains of membrane lipids contain unbranched carbon chains with even numbers of carbon atoms. Methylene groups in interrupted double bond systems are particularly sensitive to free radical attack by hydrogen atoms abstraction (Halliwell, 1989). Abstraction of a hydrogen atom from an unsaturated fatty acid is the initial step in lipid peroxidation, broadly defined as 'the oxidative deterioration of polyunsaturated lipids' (Halliwell, 1989), which leads to the formation of lipid radicals, peroxyl radicals and lipid hydroperoxides.

The addition of molecular oxygen (oxidation), which itself has low reactive radical properties, to polyunsaturated fatty acids abstracts a hydrogen atom from a carbon atom between two double bonds (-C=C-C-C=C-) leaving an unpaired electron on the carbon. The carbon radical stabilises by molecular rearrangement to form a conjugated diene (-C=C-C=C-). Under aerobic conditions the conjugated diene reacts with oxygen to give a peroxyl or peroxy radical ($RO_2\cdot$). This peroxy radical can then combine with a hydrogen atom to give a lipid hydroperoxide (HO_2^-) and further fragmentation to aldehydes, including malondialdehyde (Dormandy, 1988; Halliwell, 1989).

Two pathways of lipid peroxidation have been defined depending upon whether enzymatic oxidation or autooxidation of the unsaturated fatty acids occurs. The introduction of oxygen into unsaturated fatty acids is catalysed by oxygenases of which there are two types: the cyclooxygenases which yield endoperoxides, the precursors of prostaglandins and thromboxanes; or lipoxygenases which produce hydroperoxides, the precursors of leukotrienes, glutathione conjugates and lipoxins (Smith, 1989). These products are collectively termed eicosanoids and function as inter- and intra-cellular mediators.

Nonenzymatic peroxidation of unsaturated fatty acids can be stimulated by partially reduced oxygen and transition metals, and by hydroperoxides themselves (Girotti, 1985). Eicosanoids can also be formed in this manner, for example by autooxidation of the fatty acid arachidonic acid to give malondialdehyde (Pryor, 1976a).

Oxidants can cause peroxidation of plasma membrane lipids in many cell types and can also damage membrane proteins. Freeman and colleagues (1983) reported increased lipid peroxidation in cultured endothelial cells exposed to high oxygen levels. Also, lipid peroxidation has been detected in the mammalian lung following nitrogen dioxide exposure (Thomas, 1968), and in plasma and erythrocytes following inhalation of 0.5 ppm ozone (Buckley, 1975). Moreover, Lentz and DiLuzio (1974) found increased levels of peroxidation products in rabbit pulmonary macrophages exposed to aqueous extracts of vapour phase cigarette smoke. Products of lipid peroxidation were also detected in plasma exposed to cigarette smoke *in vitro*. (Frei, 1991).

MEASURING LIPID PEROXIDATION

The acknowledged role of lipid peroxidation in several disease states has encouraged its quantification. The quantification of the primary (hydroperoxide)

products directly, although possible, is in practice difficult because of the fleeting nature of these radicals. However, diene conjugates are maintained by lipid peroxides and most of the secondary lipid peroxidation products (Dormandy, 1988; Halliwell, 1989) and can therefore be measured. Indirect methods are also employed which analyse a secondary or end product derived from hydroperoxide transformation. The thiobarbituric acid (TBA) test is routinely employed to assess lipid peroxidation. TBA reacts with malondialdehyde (MDA) which is a volatile, low molecular weight compound. It is a side product of arachidonic acid oxidation, but also an end product of non-enzymatic, self propogated lipid degradation (Nair, 1984). This simple test involves boiling the sample with TBA in acid to form a red pigment. The amount of MDA present can then be quantified spectrophotometrically from a standard curve from the reaction of known amounts of MDA precursor with TBA under similar conditions (Bernheim, 1948).

OXIDANT INJURY AND MEMBRANE FLUIDITY

Oxidant-induced membrane lipid peroxidation is associated with a change in the physical state of the plasma membrane. Dobretsov and associates (1977), using fluorescence spectroscopy, demonstrated reduced membrane fluidity in phospholipid vesicles after radical-induced lipid peroxidation. Similarly, Rosen and colleagues (1983) found a decreased membrane fluidity for erythrocyte ghosts exposed to oxygen radicals. Moreover, although the levels of lipid peroxidation were not measured, a reduction in plasma membrane fluidity of lung cells has been observed following exposure to oxidants such as ozone (Rietjens, 1986), nitrogen dioxide (Patelk, 1988), cigarette smoke (Hannan, 1989) and free radicals generated by activated phagocytes (Ingraham, 1981; Masuda, 1990). A more rigid membrane due to lipid peroxidation could contribute to a reduction in cell deformability. Indeed, a reduction in the deformability of erythrocytes following exposure to the oxidant diamide (Fischer, 1978) was associated with a reduction in the lateral mobility of the membrane (Smith, 1982). The mechanism proposed for the altered membrane fluidity and cell deformability for erythrocytes was by cross-linking of spectrin, a component of the erythrocyte cytoskeletal network (Smith, 1982).

5.2 AIM

To determine the mechanism of the cigarette smoke-induced reduction in neutrophil deformability observed following *in vitro* exposure.

5.3 MATERIALS AND METHODS

REAGENTS

All reagents were purchased from Sigma Chemical Company, Poole, UK unless stated otherwise. Stock solutions of PMA and fMLP were prepared as described in section 2.3. Stock solutions of cytochalasin B and D were made up in DMSO and stored at -70°C. Lysophosphatidylcholine was dissolved in 3.7% formalin in PBS and kept at -20°C. N-7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) phallacidin in 100% methanol, and the fluorophores 5-N-(octadecanoyl)aminofluorescein (AF-C_{18}) and 1-hexadecanoyl-2-[N-(-7-nitrobrenz-2-oxa-1,3-diazole-4-yl)-aminohexanoyl]-sn-glycero-3-phosphocholine (NBD-PC) were obtained from Molecular Probes Inc, Cambridge, UK. NBD-phallacidin was stored at -20°C, and the fluorophores were stored at 4°C.

5.3.1 IS THE SMOKE-INDUCED INJURY OXIDANT MEDIATED?

CAN ANTIOXIDANTS PROTECT AGAINST THE SMOKE-INDUCED CHANGE IN NEUTROPHIL DEFORMABILITY?

The effect of antioxidants on the filtration of smoke exposed neutrophils was assessed by resuspending harvested neutrophils at a concentration of $1 \times 10^6.ml^{-1}$ in either PBS containing 3% BSA; PBS containing 3 μM GSH; $9 \times 10^8.ml^{-1}$ RBC; or autologous plasma (prepared by centrifugation of plasma at 3000 rpm for 20 minutes) before exposure to vapour-phase cigarette smoke and filtration as described in section 2.3.3.

To assess whether the volume in which the cells were exposed or the antioxidant concentration was more important at protecting neutrophils, cells were exposed to smoke in 2 ml and 1 ml volumes of autologous plasma and 3 μM GSH.

MEASUREMENT OF INTRACELLULAR GLUTATHIONE LEVELS

Reduced glutathione (GSH) levels in control, sham and smoke exposed neutrophils were determined using High Power Liquid Chromatography (HPLC) by Dr MME Bridgeman following the method of Cotgreave and associates (Cotgreave, 1986a). In brief, the samples were derivatised with monobromobimane (8 nM) dissolved in 50 μl acetonitrile (Fisons Scientific Equipment, Loughborough, UK) and 950 μl N-ethyl morpholine (pH 8.0) added. After 5 minutes incubation the reaction was terminated, proteins precipitated by addition of 10 ml 100% trichloroactic acid (TCA) and centrifuged at 1100 rpm for 10 minutes. The resulting supernatants were stored at

-20°C until HPLC analysis could be performed. The concentration of GSH in the cell supernatants were calculated relative to known standards of GSH.

THE EFFECT OF OXIDANT EXPOSURE ON NEUTROPHIL DEFORMABILITY

To investigate the effect of oxidant exposure on neutrophil filterability, hypochlorous acid (HOCl, 150 μM) was added to neutrophils suspended in PBS for 10 minutes at 22°C and filtered as described previously. HOCl was produced by adjusting NaOCl to pH 6.2 by drop wise addition of dilute H_2SO_4, and its concentration determined by absorbance at 235 nM (Green, 1985).

5.3.2 THE EFFECT OF SMOKE EXPOSURE ON THE NEUTROPHIL PLASMA MEMBRANE:

LIPID PEROXIDATION

The peroxidation of plasma membrane lipids was assessed for sham and smoke exposed neutrophils using the thiobarbituric acid (TBA) test to measure the concentrations of malondialdehyde (MDA)(Bernheim, 1948).

To assess MDA levels for neutrophils, 0.5 ml volumes (8×10^6 PMN.ml^{-1}) were incubated on ice with 2 ml 10% TCA for 15 minutes. Supernatants were separated by centrifugation at 3000 rpm for 10 minutes. To 1.5 mls of the supernatant an equal volume of 0.67% TBA, in distilled water was added, and the mixture boiled for 10 minutes. The mixture was allowed to cool and the absorbance was read on a Philips PU8620 spectrophotometer (Philips Scientific and Analytical Equipment, Cambridge, UK) at 535 nm. The absorbance was read for a standard curve of known concentrations of malonaldehyde-bis-(dimethyl) acetal in methanol (1:100 dilution). The standard curve was treated as the samples, with the addition of 10% trichloroactic acid (TCA) and boiling.

PLASMA MEMBRANE FLUIDITY

Plasma membrane fluidity was assessed using the Fluorescence Recovery after Photobleaching technique (FRAP)(Foley, 1986) with the kind help of Dr's JR Kusel and L Proudfoot at the Department of Biochemistry, University of Glasgow.

Two fluorescent lipid analogues, aminofluorescein (AF-C_{18}) and NBD-phosphatidylcholine (NBD-PC), were used to assess membrane fluidity. The fluorescent lipid probes were incorporated into the neutrophil membrane prior to treatment, and the lateral diffusion measured by FRAP using a laser-microscope combination with a low-powered argon laser (LEXEL model 85) with a x40 objective

lens of an Ortholux II fluorescence microscope. A spot 2 μm in diameter was bleached onto the neutrophil surface by increasing the laser power and the laser-stimulated fluorescence of the probe measured in the bleached area. The recovery of fluorescence in the bleached area was recorded on an oscilloscope (Digital Storage model 4035) and the trace analysed by computer (Hewlett Packard model 82927A). Any movement of the cell during the recovery of the bleach gave a characteristic erratic trace and these data were discarded.

PREPARATION OF THE FLUORESCENT LIPID PROBES.

NBD-PC and AF-C_{18} were the fluorescent lipids used to determine the effect of smoke exposure on lipid mobility. NBD-PC is uncharged and therefore inserts into the hydrophobic leaflet of the plasma membrane, joining onto hydrophobic fatty acids. The AF-C_{18} fluorophore consists of a hydrophilic headgroup and locates in the outer leaflet of the plasma membrane, with it's hydrophobic fatty acid (C_{18}) tail inserted into the hydrophobic region. Although incorporated into the membrane in a slightly different manner, both these fluorescent lipids provide a measure of lipid mobility in the outer leaflet of the plasma membrane.

AF-C_{18} (Figure 5.1a): The dry powdered form of the fluorescent lipid analogue AF-C_{18} was dissolved in ethanol at a concentration of 1 $mg.ml^{-1}$. The stock solution was diluted in PBS to give a final concentration of 10 $\mu g.ml^{-1}$.

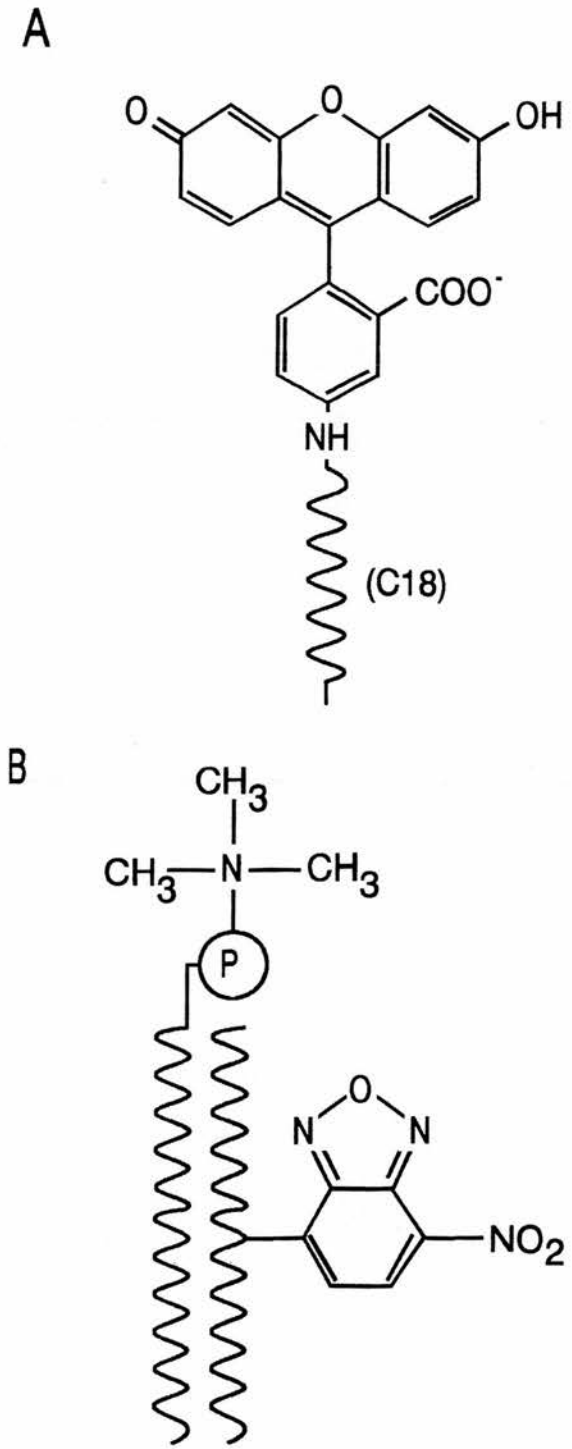
NBD-PC (Figure 5.1b): Fifty micrograms of NBD-PC was dissolved in 200 μl choloroform and added to 1 mg egg lecithin (non-fluorescent phosphatidylcholine) in 50 μl chloroform. After thorough mixing the solution was dried under nitrogen. Ethanol was added to the dry lipids and mixed gently. The lipid mixture was then slowly injected into a stirring 1 ml volume of PBS at 35°C over 2 minutes. The liposomes were dialysed overnight at 4°C against PBS to remove any free fluorophores not in liposomes.

The fluorophores were incorporated into the neutrophil plasma membrane by incubation at 37°C for 10 minutes in the dark. The cells were washed twice to remove any residual fluorescence and resuspended in PBS containing 0.5% BSA at a concentration of 1 x $10^6.ml^{-1}$. A drop of sham or smoke exposed neutrophil suspensions was placed on a glass slide and the cells immobilised using a glass coverslip. Photobleaching was performed by Dr Lorna Proudfoot on randomly selected neutrophils at 37°C. The laser power was increasing by 10^4 for 50

milliseconds then returned to normal and measurement resumed. The brief increase in power is set to irreversibly bleach about 50% of the probe in the spot. If there is no freedom of motion of molecules in and out of the spot, the fluorescence intensity will remain at this level ad infinitum. If, however, there is complete freedom to diffuse in and out of the spot, the fluorescence intensity will return to the prebleach level (as indicated in Figure 5.2). An intermediate condition can also occur where only a fraction of the probe is free to diffuse, and partial recovery is observed (Axelrod, 1976; Koppel, 1976). FRAP thus gives two measures of fluidity: the fraction or percent of the probe that is mobile in the plasma membrane (percentage recovery), and the diffusion coefficient of the fraction. The percentage recovery is calculated from the estimated signal at infinite time after photobleaching (Foley, 1986). The lateral diffusion coefficient is calculated from the kinetics of the recovery curve (Figure 5.2), to give a measure of the speed of recovery, using the equation:

$$\text{Lateral Diffusion } (D_L) = W^2/4t_{1/2} \times \gamma$$

where W is the spot size (radius), $t_{1/2}$ is the half-time taken for full (possible) recovery, and γ is the related constant for the viscosity measurement (Axelrod, Koppel et al., 1976).



FIGURES 5.1a and b
Structures of the two fluorescent lipid probes used to measure plasma membrane fluidity: (a) 5-N-(octadecanoyl)-aminofluorescein attached to an 18 carbon fatty acid (AF-C_{18}) and (b) 1-hexadecanoyl-2-[N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl] linked to phosphatidylcholine (NBD-PC).

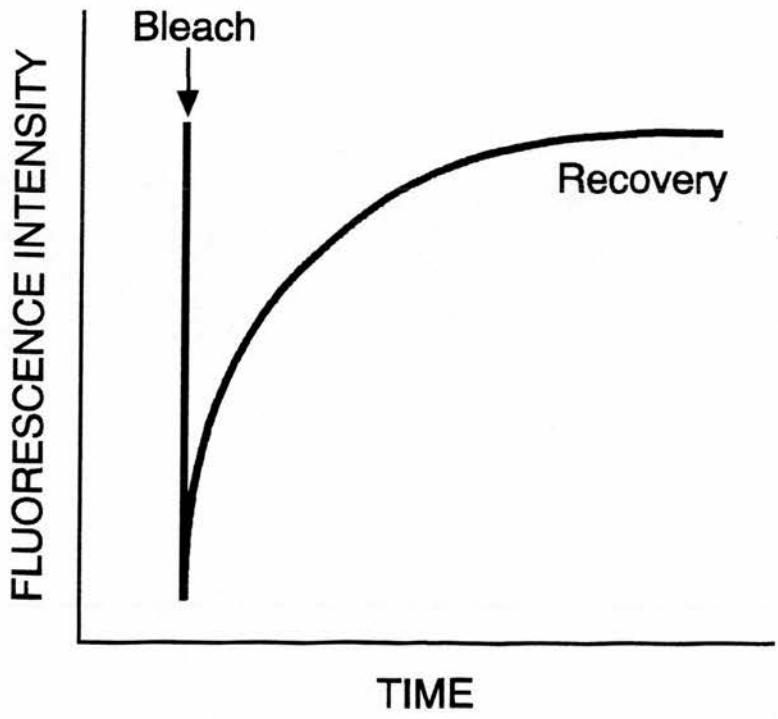


FIGURE 5.2
Schematic illustration of the principle of the Fluorescence Recovery After Photobleaching technique. Fluorophores incorporated in the plasma membrane are bleached by a pulse of high laser power. Subsequent reappearance of fluorescence in the bleached area is monitored as a measure of lateral membrane fluidity.

5.3.3 THE EFFECT OF SMOKE EXPOSURE ON THE NEUTROPHIL CYTOSKELETON:

CYTOSKELETAL INHIBITORS

The influence of the cytoskeleton on smoke-induced changes in neutrophil deformability was investigated by the addition of specific inhibitors for microfilament formation (the cytochalasins)(Brown, 1979) or microtubules (colchicine)(Olmsted, 1973). Cytochalasin B (5 $\mu g.ml^{-1}$), cytochalasin D (0.6 $\mu g.ml^{-1}$) or colchicine (30 $\mu g.ml^{-1}$ made up fresh in PBS) were added to sham, smoke-exposed, or stimulated cells. Colchicine was added for 20 min at 22°C before sham, smoke exposure or fMLP stimulation ($10^{-7}M$); whereas cytochalasin B and D were added after sham or smoke exposure just prior to filtration.

QUANTIFICATION AND VISUALISATION OF INTRACELLULAR F-ACTIN:

FLUORESCENCE LABELLING OF F-ACTIN

Changes in the F-actin component of the cytoskeleton were assessed by fluorescently labelling neutrophils with NBD phallacidin by the method of Howard and Meyer (1984).

Neutrophils in PBS containing 0.5% BSA were either sham or smoke exposed, or stimulated with PMA. As albumin was found to interfere with the permeabilisation of the cells, the treated cells were washed once and resuspended in PBS (1.1×10^6 $PMN.ml^{-1}$) prior to staining with NBD phallacidin using a two-step technique. The cells were fixed with 3.7% formalin in double strength PBS and permeabilised with 100 $\mu g.ml^{-1}$ lysophospatidylcholine for 15 minutes at ambient temperature. The neutrophils were then stained by addition of $1.65 \times 10^{-7}M$ NBD phallacidin and 20 minutes incubation at 37°C. The cells were subsequently washed and resuspended in PBS and stored in the dark at 4°C.

MEASUREMENT OF F-ACTIN BY FLOW CYTOMETER

The fluorescence emission of individual cells was quantitatively assessed using an EPICS CS flow cytometer (Coulter Electronics, UK) as a measure of F-actin content. The flow cytometer was calibrated daily using polystyrene beads (Immunocheck alignment fluorospheres, 10 μm diameter; Coulter Electronics Ltd., Luton, UK). Fluorescence was excited by 500 mW laser at 488 nm, and detected using a 560 nm short pass emission filter. Neutrophils were gated by their forward and side scatter light characteristics. Histograms of cell number versus linear fluorescence intensity were recorded for 5000 cells for each treatment per experiment. As the F-actin content is proportional to the fluorescence intensity (Howard and Meyer, 1984), the data was expressed as the linear fluorescence channel number (FI). The mean and

standard deviation of the fluorescence intensity distributions were obtained by analysis using a EPICS CS Integral Data Acquisition System (Coulter Electronics, UK).

Neutrophil actin content was assessed following sham or smoke exposure. Cytochalasin B (5 $\mu g.ml^{-1}$) was added to duplicate samples of sham and smoke exposed neutrophils to confirm actin polymerisation. As a comparison for the extent of actin polymerisation caused by smoke exposure, neutrophils were stimulated with PMA (1.0 $\mu g.ml^{-1}$) or fMLP ($10^{-7}M$) for 10 minutes and the cellular actin content assessed as above.

FLUORESCENCE MICROSCOPY

Qualitative assessment of the cellular F-actin content was assessed in cells which were sham or smoke exposed, and in smoke exposed cells treated with cytochalasin B (5 $\mu g.ml^{-1}$) prepared as described above. The cells were pelleted and resuspended in PBS containing 100 $mg.ml^{-1}$ 1,4-diazabicyclo (2.2.2) octane (DABCO) to minimise quenching of fluorescence when exposed to the laser. A drop of the cell suspension was placed on a glass slide and covered with a glass coverslip which was sealed using clear nail varnish. The cells were observed using a Zeiss LSM10 confocal scanning fluorescence microscope with the help of Mr Scott Cunningham, Department of Pathology, Edinburgh University. Preliminary observations were made with normal white light using a x40 water immersion lens. Incident argon laser light (488 nm) was then selected and the cells were scanned employing zoom factors of 80 or 120. Photographs of the cells were taken from the monitor screen using a Contax camera with 50 ASA film.

5.3.4 STATISTICAL ANALYSIS

The statistical analysis performed on the data in this chapter were as detailed in chapter 2.

5.4 RESULTS

5.4.1 THE CIGARETTE SMOKE -INDUCED INJURY TO NEUTROPHILS IS OXIDANT MEDIATED

ANTIOXIDANTS PROTECT AGAINST THE SMOKE-INDUCED REDUCTION IN NEUTROPHIL DEFORMABILITY

Smoke exposed neutrophils suspended in plasma had lower filtration pressures than neutrophils exposed to smoke when suspended in buffer (Figure 5.3). Furthermore, the antioxidants albumin and GSH, at concentrations present in plasma (3% and 3 μM respectively)(Bridgeman, 1991; Cantin, 1987), also reduced the effect of smoke exposure on filtration pressures (Figure 5.3). Similarly, the presence of low concentrations of erythrocytes ($9 \times 10^8.ml^{-1}$) ameliorated the effect of smoke exposure on neutrophil filtration pressures (Figure 5.3).

Interestingly the protective effect of plasma was reduced when cells were exposed to smoke suspended in a smaller volume (1 ml)(Figure 5.4). However, neutrophils exposed to smoke in the presence of 3 μM GSH, but in a 1 ml volume, did not develop higher filtration pressures than neutrophils exposed to smoke in a 2 ml volume containing 3 μM GSH (P_6 2 ml 9.2 ± 4.2 cm H_2O; P_6 1 ml 9.2 ± 4.5 cm H_2O; n=6, $p>0.05$).

INTRACELLULAR GLUTATHIONE LEVELS

GSH levels in neutrophils decreased following exposure to cigarette smoke *in vitro* compared with sham exposed and control cells (sham exposed 3.2 ± 1.7 $nM.10^6$ PMN, smoke exposed 0.5 ± 0.4 $nM.10^6$ PMN; n=6, $p<0.01$).

NEUTROPHIL FILTERABILITY FOLLOWING EXPOSURE TO HOCl

Addition of the oxidant HOCl reduced neutrophil filterability (Figure 5.5).

5.4.2 *IN VITRO* SMOKE EXPOSURE DOES NOT AFFECT THE NEUTROPHIL MEMBRANE:

LIPID PEROXIDATION

Lipid peroxidation, as measured by MDA levels, was not detected when a 2 ml volume of neutrophils (2×10^6) was exposed to cigarette smoke (sham exposed 0.79 ± 0.15 $nmol\ MDA.ml^{-1}$, smoke exposed 1.22 ± 0.6 $nmol\ MDA.ml^{-1}$; n=6, $p>0.05$). However, exposure of neutrophils to the same dose of smoke, but suspended in a smaller volume of buffer (0.5 ml), did increase MDA levels (Figure 5.6). The levels of MDA following sham exposure of neutrophils were no different from control cells

(control 0.78 ± 0.4 nmol MDA.ml^{-1}, sham exposed 0.79 ± 0.15 nmol MDA.ml^{-1}; n=6, p>0.05).

PLASMA MEMBRANE FLUIDITY FOLLOWING *IN VITRO* SMOKE EXPOSURE

Representative FRAP curves for neutrophils following (a) sham exposure and (b) smoke exposure are shown in Figure 5.7. Plasma membrane fluidity of smoke exposed neutrophils was not significantly different from sham exposed neutrophils measured by the lateral diffusion of either fluorophore (Figures 5.8a and b). Likewise, the percentage recovery of fluorescence in the bleached area was not significantly affected by smoke exposure (AF-C_{18}, sham exposed 34.5 ± 17.3%, smoke exposed 32.1 ± 11.1%; n=15, p>0.05: NBD-PC, sham exposed 31.71 ± 15.8% smoke exposed 29.7 ± 10.8%; n=12, p>0.05).

5.4.3 CIGARETTE SMOKE EXPOSURE ALTERS THE NEUTROPHIL CYTOSKELETON

THE EFFECT OF CYTOSKELETAL INHIBITORS ON SMOKE EXPOSED NEUTROPHIL FILTERABILITY

The filtration pressures developed by control neutrophils, not exposed to smoke, was unaltered by the addition of cytochalasin B (CB) or colchicine (COL) (pressure developed after 6 min filtration (P_6) control 4.1 ± 0.8 cm H_2O, P_6 CB 4.3 ± 1.4 cm H_2O; n=6, p>0.05: P_6 control 8.1 ± 1.8 cm H_2O, P_6 COL 8.1 ± 2.9 cm H_2O; n=8, p>0.05). Likewise pre-incubation of neutrophils with colchicine prior to smoke exposure had no effect on the filtration pressures (Figure 9a). Likewise, the filtration pressures developed by fMLP stimulated neutrophils were similarly unaffected by pre-incubation with colchicine (fMLP 8.2 ± 3.8 cm H_2O, fMLP + COL 7.2 ± 3.6 cm H_2O; n=6, p>0.05). Addition of colchicine to neutrophils post cigarette smoke exposure did not alter cell filtration pressures (data not shown). In contrast, addition of cytochalasin B or cytochalasin D (CD) abolished the smoke-induced filtration pressures (CB Figure 5.9b: CD P_6 smoke exposed 14.3 ± 4.4 cm H_2O, P_6 smoke exposed + CD 9.9 ± 2.0 cm H_2O; n=4, p<0.01).

QUANTIFICATION AND VISUALISATION OF NEUTROPHIL ACTIN CONTENT

The increase in the actin content of neutrophils following *in vitro* smoke exposure was confirmed and quantitated by fluorescence staining of F-actin with NBD-phallacidin. A plot of cell number versus linear fluorescence for untreated neutrophils resulted in a histogram (Figure 5.10a) from which the mean F-actin content for the sample was determined (mean channel linear fluorescence intensity

(FI) 29.6 ± 5.5, n=8). A small, non-significant increase in mean fluorescence intensity was observed following sham exposure (FI sham exposed 35.2 ± 11.8, n=8, $p>0.05$). However, a marked increase in fluorescence intensity was observed following *in vitro* smoke exposure (FI 115.4 ± 28.7, n=8, $p<0.01$), although a non-responding subpopulation of cells was also evident (FI 31.4 ± 5.7, n=8, $p>0.05$). Representative histograms for control, sham exposed, and smoke exposed neutrophils are shown in Figures 5.10a - d. The mean values for 8 experiments are summarised in Figure 5.11.

In comparison, stimulation of neutrophils with PMA did not significantly alter the fluorescence intensity compared with control cells (FI control 29.6 ± 5.4, PMA 33.8 ± 9.9, n=8, $p>0.05$), whereas a marked shift to an increased mean fluorescence was noted following fMLP stimulation for 10 minutes (FI control 42.7 ± 13.3, fMLP 68.3 ± 7.4; n=5, $p<0.01$).

Inhibition of F-actin by cytochalasin B was demonstrated for smoke exposed and stimulated cells by a return to mean fluorescence levels similar to sham exposed neutrophils following treatment with cytochalasin B (FI smoke exposed 115.4 ± 28.7, smoke exposed + CB 36.5 ± 5.7; n=8, $p<0.01$), as illustrated in Figure 5.10d, with mean values for 8 experiments shown in Figure 5.11. However, addition of cytochalasin B to sham exposed neutrophils caused an increase in fluorescence intensity (sham exposed 39.3 ± 17.0, sham exposed + CB 48.0 ± 11.9; n=4, $p<0.05$).

Fluorescence micrographs revealed an increased fluorescence intensity in the subcortical region of neutrophils exposed to smoke, which was not evident in sham exposed cells (Figure 5.12b and 5.12a respectively). F-actin in cytochalasin B treated smoke exposed neutrophils was similar to sham exposed neutrophils with little cortical staining (Figure 12c).

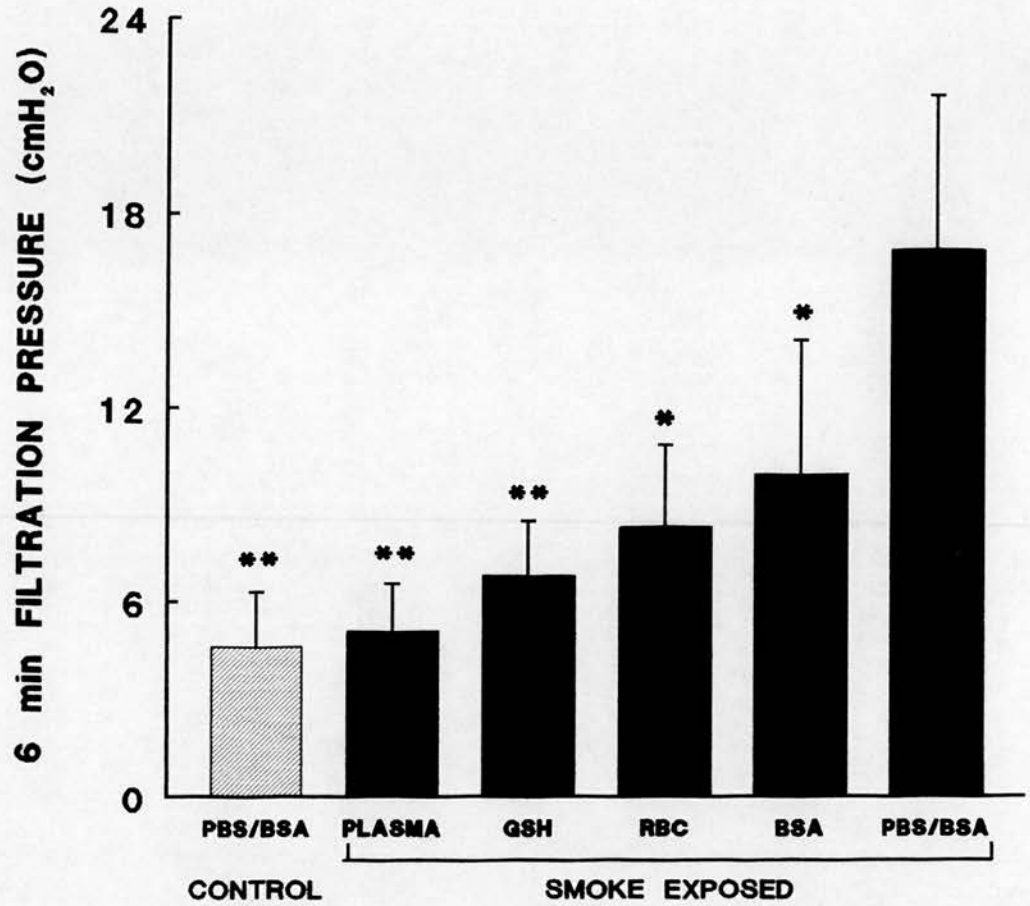


FIGURE 5.3
The pressures developed following 6 minutes filtration (P_6) of neutrophils exposed to cigarette smoke (closed bars) whilst suspended in PBS containing 0.5% BSA (PBS/BSA); 3% BSA; 9 x 10^8 ml^{-1} RBC; 3 μM GSH; or in autologous plasma. Compared with sham exposed neutrophils (hatched bar), cells exposed to cigarette smoke developed greater filtration pressures. All treatments significantly reduced the filtration pressures compared with neutrophils exposed to smoke in PBS/BSA. Mean values with error bars representing 1 SD for 7 experiments are shown. Compared with neutrophils smoke exposed in PBS/BSA, * $p<0.05$, ** $p<0.01$.

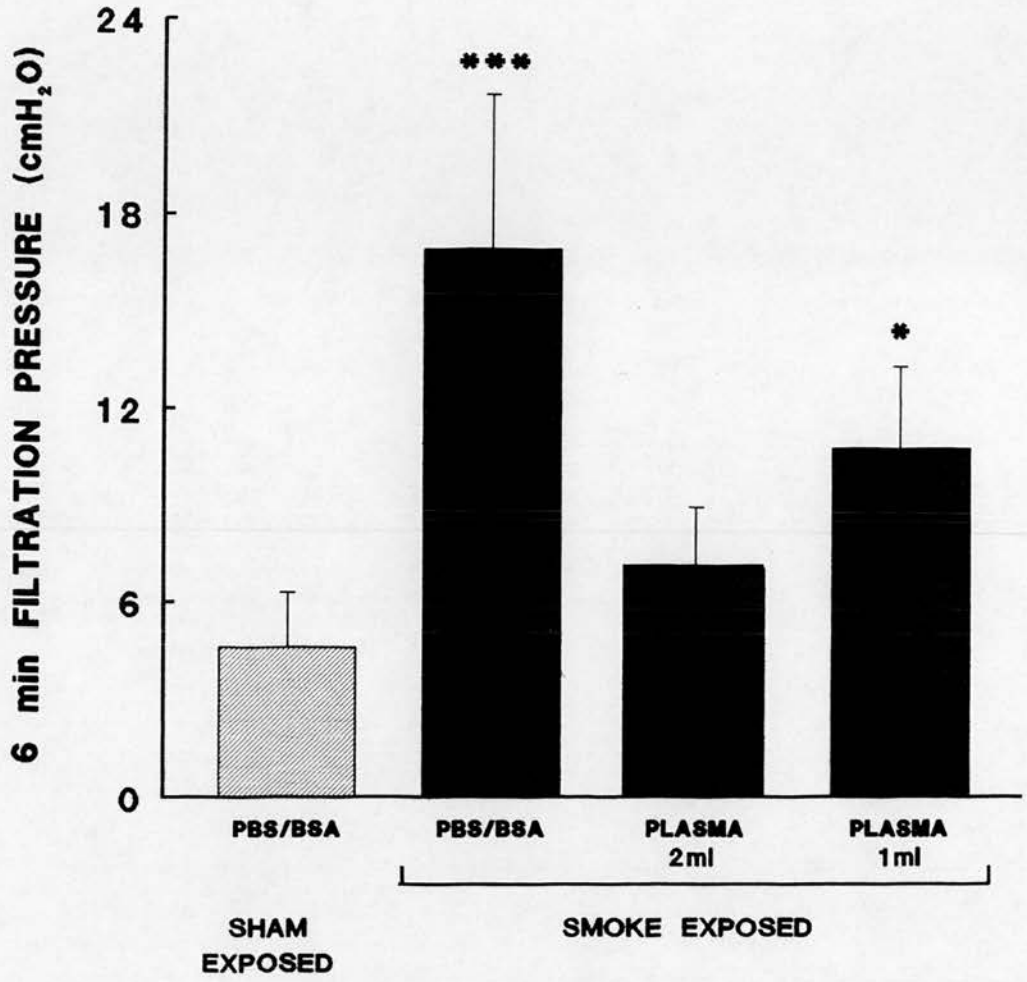


FIGURE 5.4
Filtration pressures at 6 minutes (P_6) for neutrophils exposed to cigarette smoke (solid bars) suspended in PBS/BSA, or in an equivalent (2 ml) or reduced (1 ml) volume of autologous plasma. Neutrophils (2 ml) exposed to smoke in the presence of plasma developed lower filtration pressures similar to sham exposed neutrophils (hatched bar). Exposure to smoke in a reduced volume provided some protection compared with exposure suspended in PBS/BSA, but pressures were significantly higher than sham exposed neutrophils. Mean values with bars representing 1 SD are shown for 7 experiments. Compared with sham exposed neutrophils, * $p<0.05$, *** $p<0.001$.

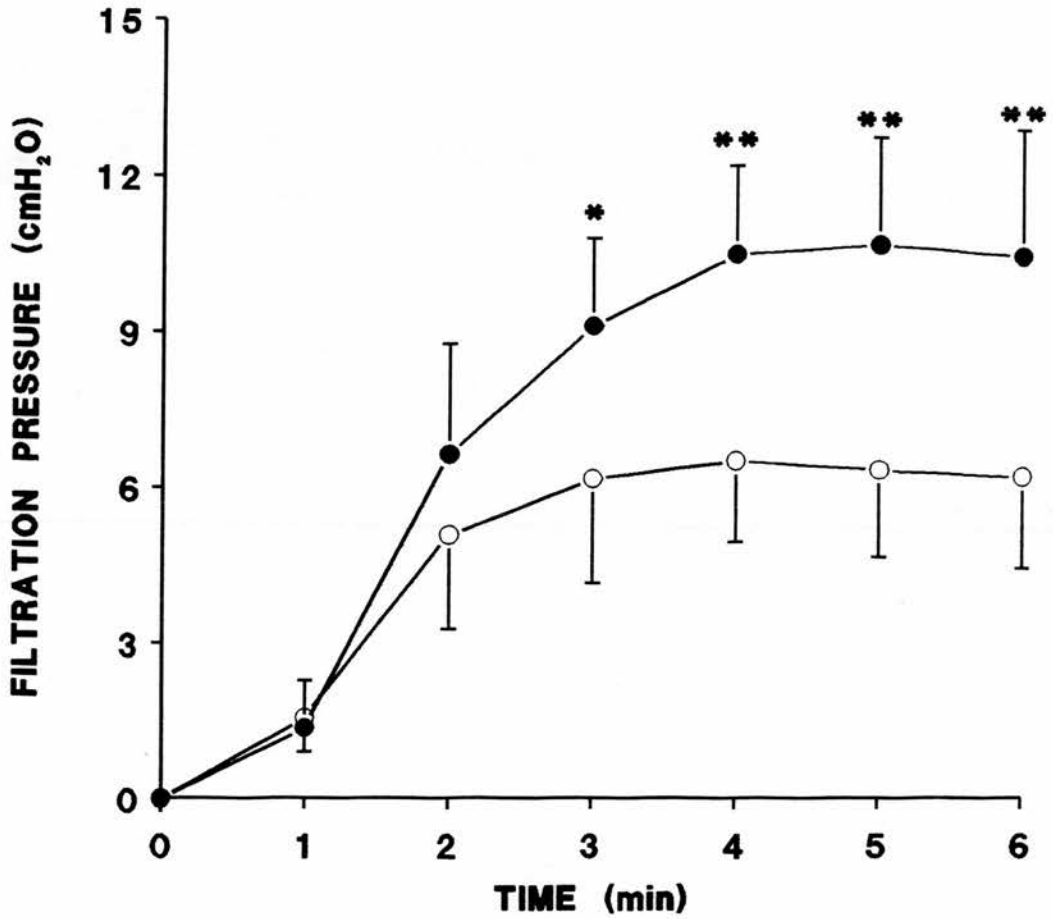


FIGURE 5.5
The filtration pressures of neutrophils after incubation with 150 μM hypochlorous acid (HOCl). Neutrophils exposed to 150 μM HOCl (●) developed higher filtration pressures than control neutrophils (O). Mean values for 7 experiments are shown with error bars representing 1 SD. Compared with control neutrophils, * $p<0.05$, ** $p<0.01$.

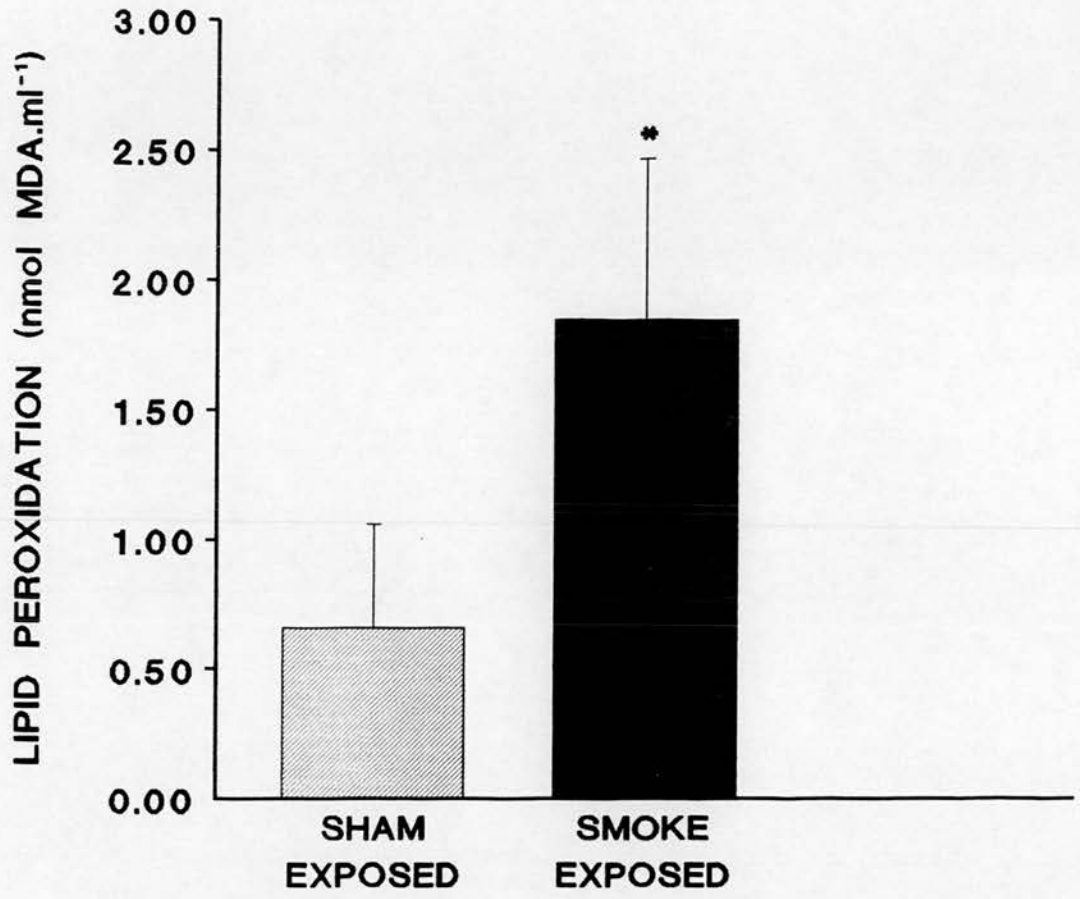
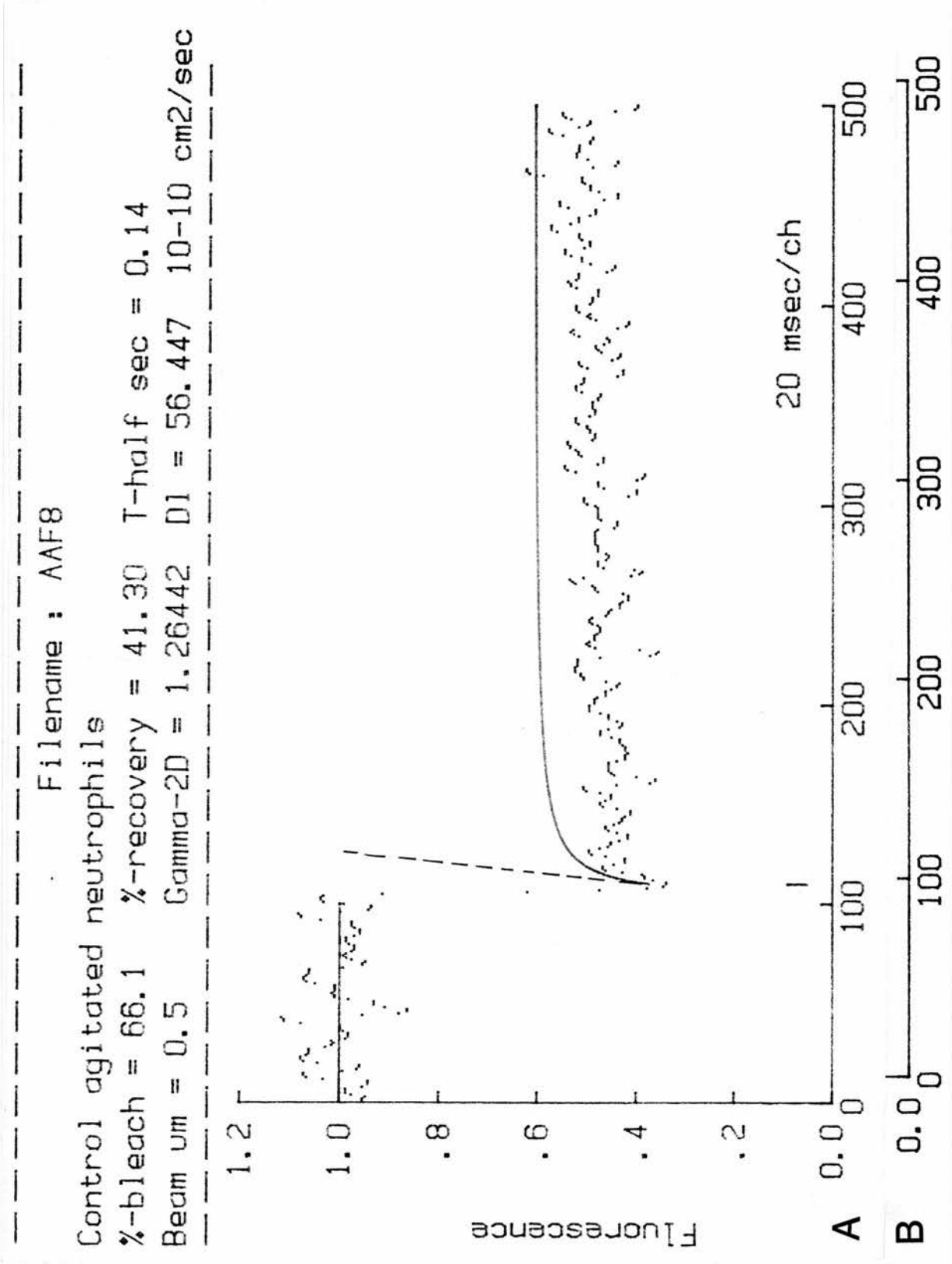
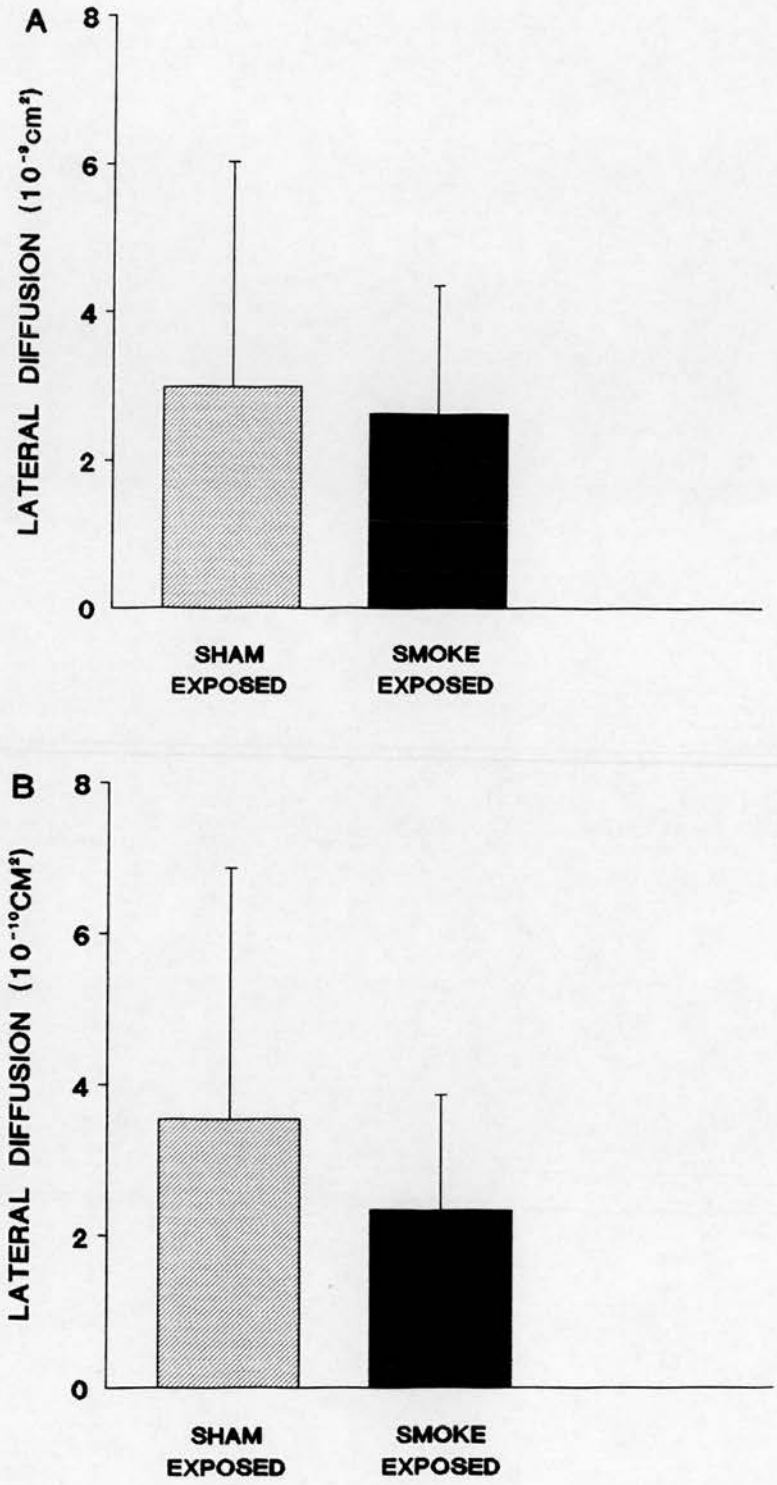


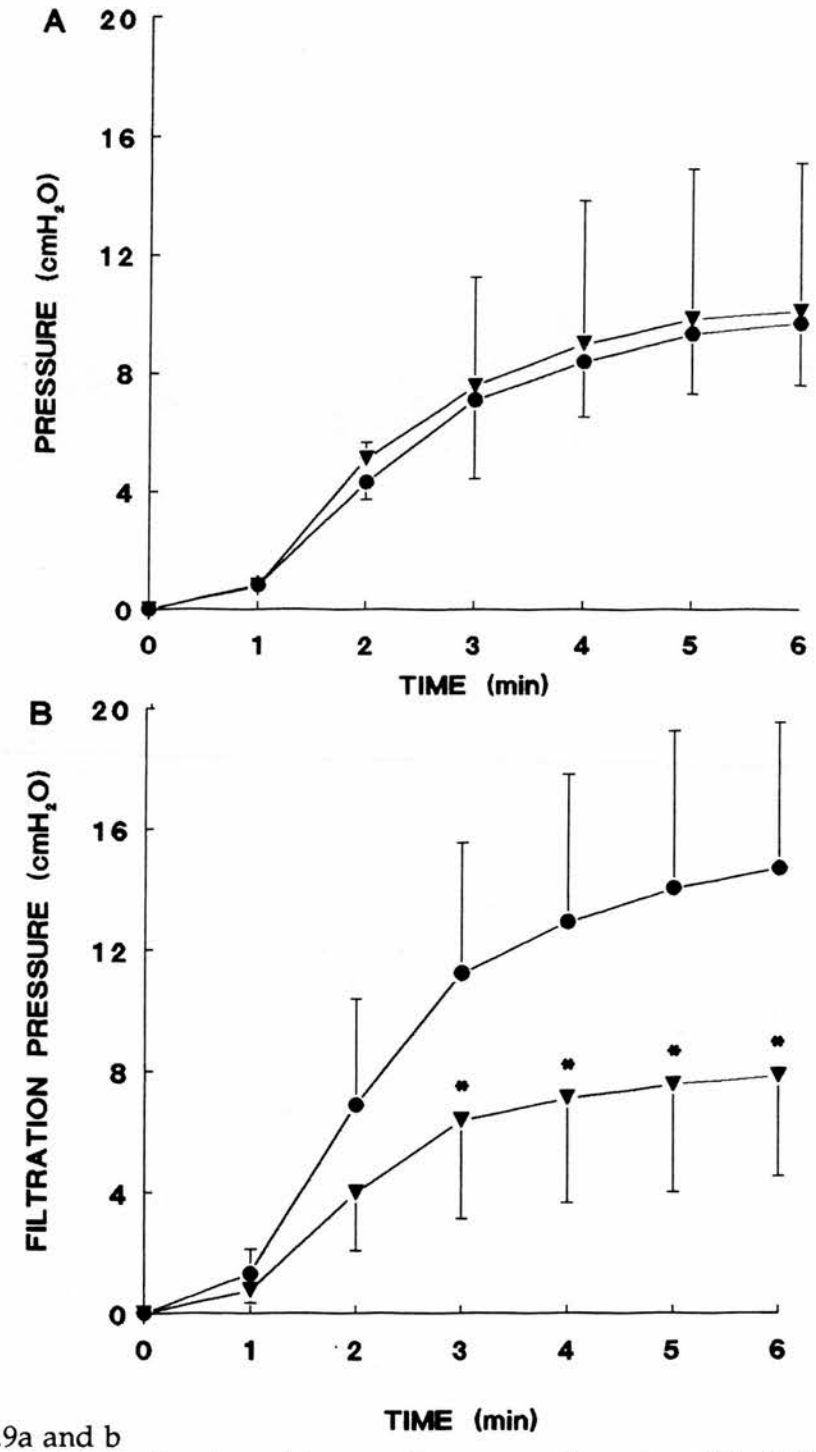
FIGURE 5.6
Malondialdehyde (MDA) levels determined from sham (hatched bar) and smoke exposed (closed bar) neutrophils ($8 \times 10^6.ml^{-1}$) as a measure of lipid peroxidation. A significant increase in the levels of MDA was evident following *in vitro* smoke exposure of a 0.5 ml volume of neutrophils. Mean values with error bars representing 1 SD are shown for 7 experiments. Compared with sham exposed, * $p<0.05$.



FIGURES 5.7a and b
Representative fluorescence recovery curves for (a) sham and (b) smoke exposed neutrophils obtained following photobleaching. The broken line represents the extrapolation from the first moment of the curve from which the diffusion coefficient (D_L) is calculated.

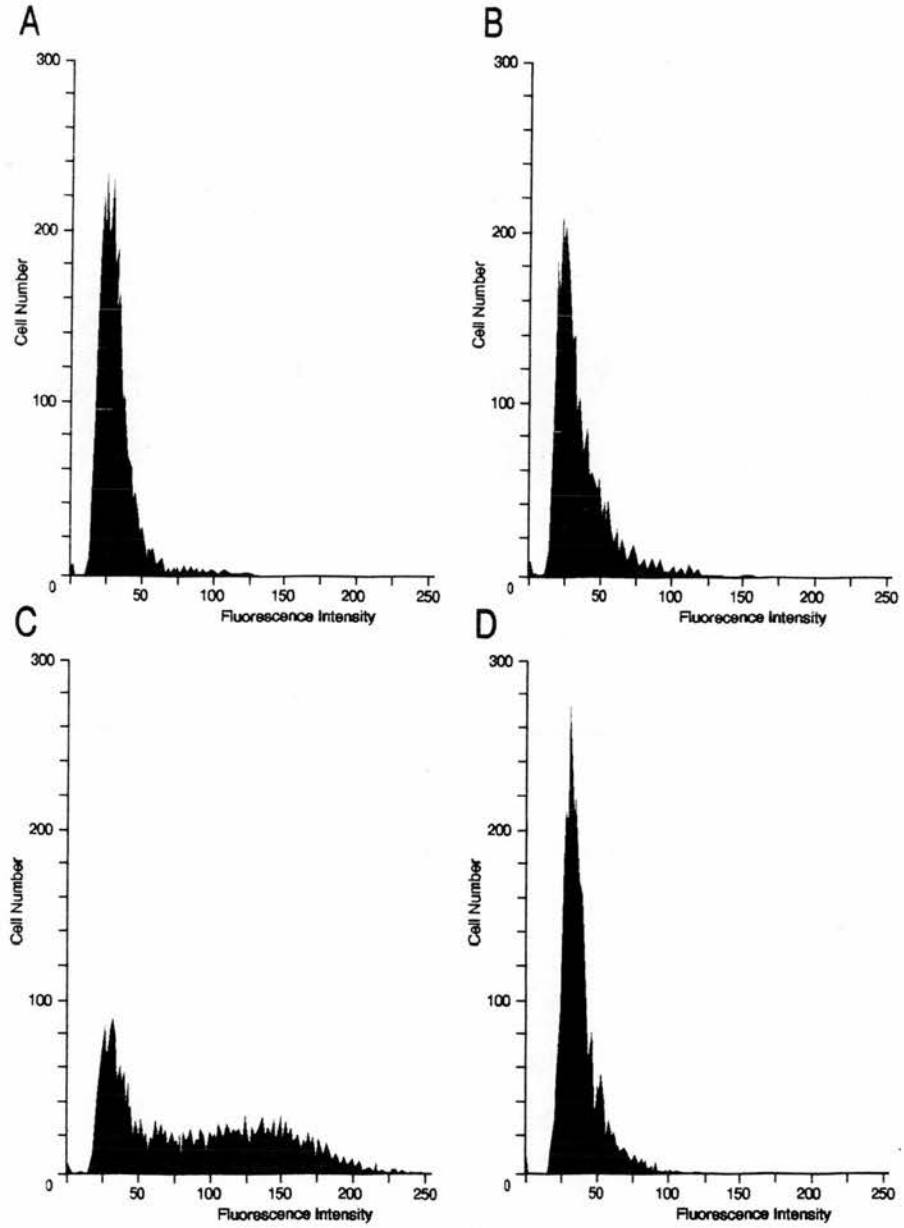


FIGURES 5.8a and b
Lateral diffusion of plasma membrane lipids for sham (hatched bar) and smoke exposed (closed bar) neutrophils measured using the fluorophores (a) AF-C_{18} and (b) NBD-PC. The lateral diffusion of membrane lipids was unaltered by smoke exposure. Mean values for (a) 15 cells and (b) 12 cells for each treatment are shown with error bars representing 1 SD.



FIGURES 5.9a and b

Filtration pressures developed by smoke exposed neutrophils (●) and smoke exposed neutrophils either (a) preincubated with 30 $\mu g.ml^{-1}$ colchicine for 10 minutes (n=8) or (b) 5 $\mu g.ml^{-1}$ cytochalasin B added post exposure (n=7)(▼). Preincubation with colchicine had no effect on filtration pressures developed by smoke exposed neutrophils. In contrast, addition of cytochalasin B abolished the increased filtration pressures observed for smoke exposed neutrophils. Mean values are shown with error bars representing 1 SD. Compared with control neutrophils, *$p<0.05$.



FIGURES 5.10a - d
Representative histograms of linear fluorescence intensity for neutrophils stained NBD phallacidin, a fluorescent probe specific for filamentous actin. Treatments shown are (a) control, (b) sham exposed, (c) smoke exposed neutrophils and (d) smoke exposed neutrophils with cytochalasin B. A significant increase in fluorescence intensity was observed following smoke exposure for a subpopulation of the neutrophils. The addition of cytochalasin B post smoke exposure attenuated the increase in fluorescence, confirming the increase in actin polymerisation as a result of smoke exposure and the specificity of the NBD phallacidin label.

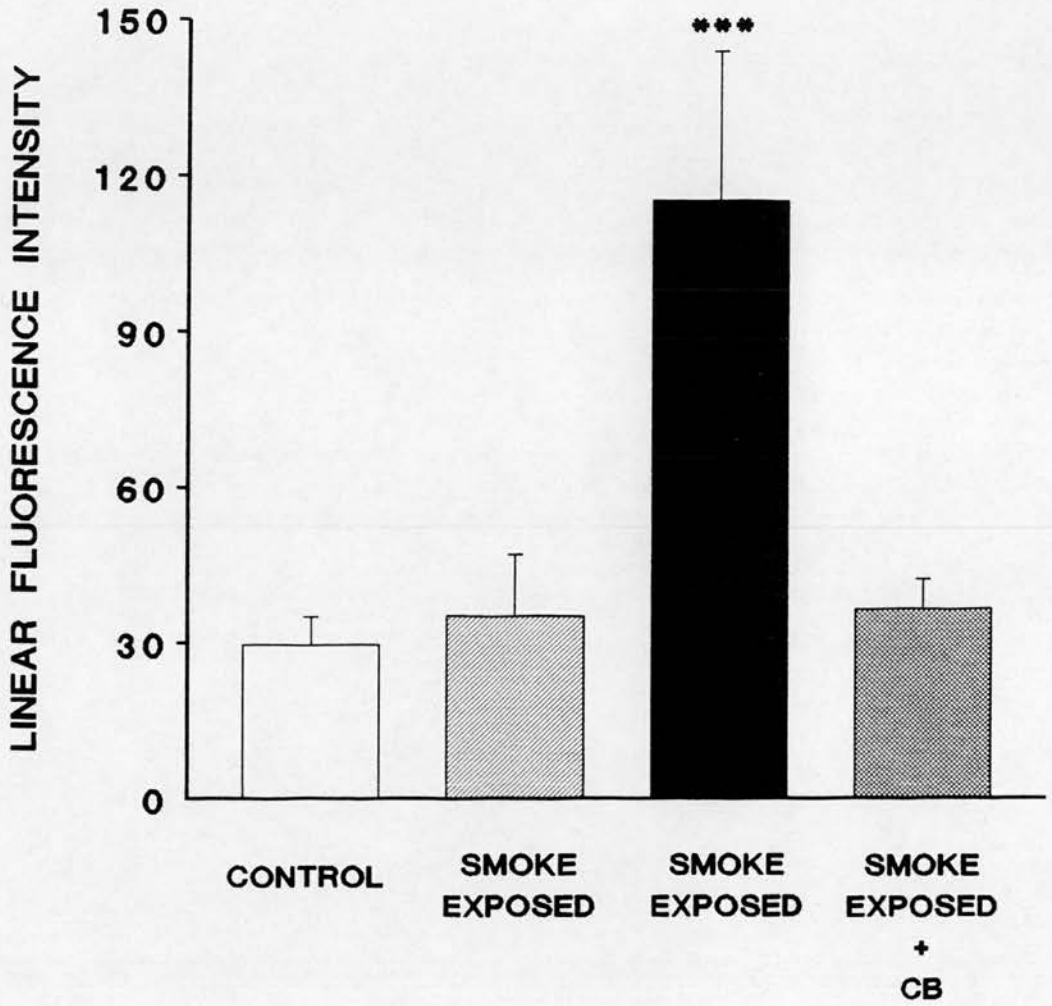
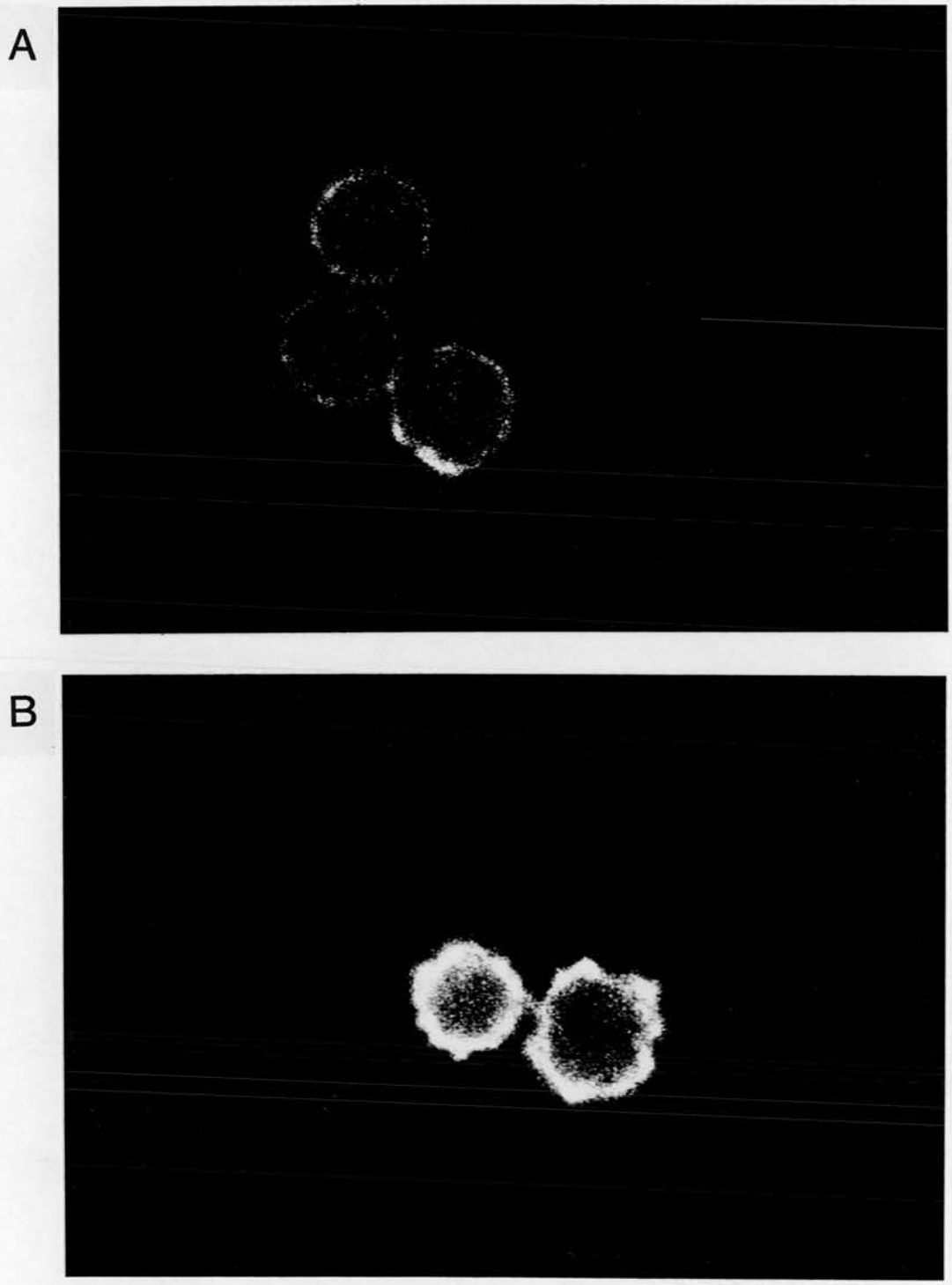


FIGURE 5.11
The effect of *in vitro* smoke exposure on neutrophil filamentous actin (F-actin) content for control (open bar), sham (hatched bar) and smoke exposed neutrophils (closed bar), and smoke exposed neutrophils following the addition of 5 $\mu g.ml^{-1}$ cytochalasin B (CB)(crossed bar). A significant increase in actin polymerisation was observed following smoke exposure, which was abolished by the subsequent addition of cytochalasin B. Mean values for 8 experiments are shown, with error bars representing 1 SD. Compared with sham exposed neutrophils, *** $p<0.001$.



FIGURES 5.12a - c
Localisation of NBD phallacidin, specific for filamentous actin, in (a) sham exposed, (b) smoke exposed neutrophils, and (c) smoke exposed neutrophils following subsequent addition of cytochalasin B (see following page). A thin rim of cortical staining was evident for sham exposed neutrophils (a). In contrast a dramatic enhancement of NBD phallacidin fluorescence was evident in smoke exposed cells (b) (x4.8 x 10^3 magnification), which was abolished by addition of cytochalasin B (c) (x3.2 x 10^3 magnification).

C

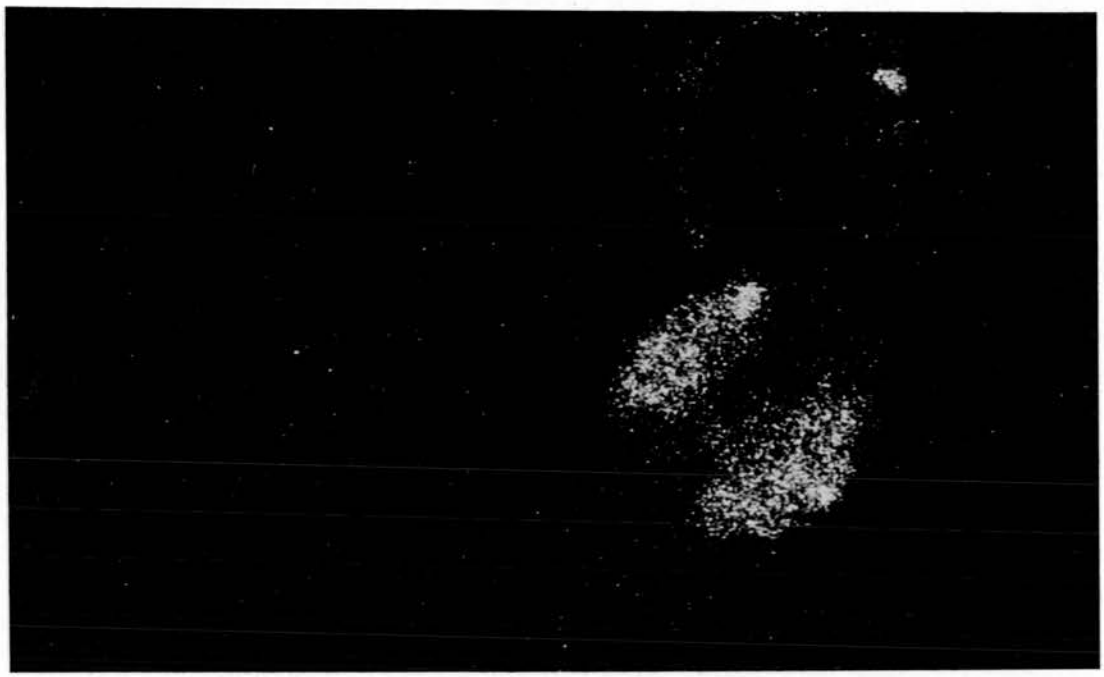


FIGURE 5.12 CONTINUED
Fluorescence micrograph of smoke exposed neutrophils subsequently treated with cytochalasin B. (see previous page for legend.)

5.5 DISCUSSION

The data in this chapter suggest an increase in the filamentous form of actin as the mechanism for the smoke-induced change in neutrophil deformability, which appeared to be oxidant mediated. Increased F-actin was implicated as addition of the specific F-actin inhibitors, cytochalasins B and D abolished the smoke-induced increase in filtration pressures. These observations are in agreement with other studies which report enhanced retention of neutrophils in micropore membranes as a result of increased actin polymerisation (Frank, 1990a; Pecsvarady, 1992; Worthen, 1989). Moreover, Smith and co-workers (1986) also reported a reduced filterability of smoke exposed hamster macrophages, associated with microfilament formation.

5.5.1 ACTIN POLYMERISATION

NEUTROPHIL ACTIVATION AND ACTIN POLYMERISATION

Neutrophil shape change and actin polymerisation are believed to be related as they occur concomitantly following cell activation (Coates, 1992; Frank, 1990a; Howard, 1985; Watts, 1991). In order to migrate through the endothelium of vessel walls, undergo chemotaxis, and to phagocytose microbes, the neutrophil must reorganise it's cytoskeleton and undergo shape change. Hence, the relationship between actin polymerisation and cell shape has been well studied, particularly for the synthetic bacterial peptide fMLP (Belloc, 1990; Howard, 1985; Packman, 1990; Wallace, 1984; Watts, 1991).

Stimulation with fMLP induces a peak increase in cellular F-actin in human neutrophils 10 - 30 seconds after it's addition (Frank, 1990a; Howard, 1985; Wallace, 1984; Westlin, 1992), with a gradual depolymerisation although still remaining above baseline levels at 30 minutes when using fMLP concentrations greater than 1 nM (Howard, 1985; Wallace, 1984). The rate of polymerisation, and the extent of polymerisation and depolymerisation are fMLP-dose dependent (Howard, 1985; Wallace, 1984). Cano and colleagues (1991) showed that the chemoattractant-induced increase in F-actin levels in neutrophils resulted from an increase in filament number, either by severing of existing filaments or *de novo* synthesis of new filaments, without any change in filament length.

A similar rapid response as with fMLP stimulation was observed when neutrophils were stimulated with leukotriene B_4 (LTB_4)(Westlin, 1992), interleukin-8 (IL-8)(Westlin, 1992), platelet activating factor (PAF)(Bochsler, 1992; Packman, 1990; Westlin, 1992), zymosan activated serum (ZAS)(Bochsler, 1992), or the fifth fragment of the complement factor (C_{5f})(Bochsler, 1992; Westlin, 1992).

PMA stimulation, in contrast, produced slower actin polymerisation (Howard , 1987) with F-actin content peaking several minutes after the addition of PMA (Belloc, 1990; Roos, 1987), although Sheterline and associates (1986) reported a rapid (30 seconds) increase in actin polymerisation following phorbol ester stimulation. However, in their studies a different phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was used on neutrophils obtained from pigs which may explain the discrepancy. The PMA-induced increase in F-actin, in contrast to chemoattractant stimulation, was sustained for up to 30 minutes (Belloc, 1990; Roos, 1987).

CHANGES IN NEUTROPHIL F-ACTIN AS A RESULT OF SMOKE EXPOSURE

Direct measurement of intracellular F-actin using fluorescence staining confirmed an increase in F-actin content in a subpopulation of neutrophils exposed to vapour phase cigarette smoke (Figure 5.11). The extent of actin polymerisation of smoke exposed neutrophils was markedly greater (3.3 fold) than the increase measured following fMLP stimulation (1.6 fold). However, the fMLP-induced F-actin levels were assessed 10 minutes after addition of the stimulant, hence the peak response could have been missed. PMA-stimulation did not significantly alter F-actin levels in neutrophils, as found by Bengtsson (1986), but is in contrast to the significant actin polymerisation reported by several others (Belloc, 1990; Roos, 1987; Sheterline, 1986). However, Howard and Wang (1987) found that high concentrations of PMA were required for even minimal changes in F-actin compared to the concentrations of PMA required to produce functional responses. A reduction in cell deformability can result from only a small increase in the amount of polymerised actin (Frank, 1990a; Pecsvarady, 1992), which may explain why a reduction in neutrophils deformability was observed with PMA-stimulation (section 2.4.3) without a significant increase in F-actin.

Addition of the F-actin inhibitors, cytochalasin B and D abolished the smoke-induced increase in F-actin levels in neutrophils (cytochalasin B: Figures 5.10d and 5.11), as is also the case with the actin polymerisation which accompanies cell activation (Belloc, 1990; Cano, 1991; Frank, 1990a; Pecsvarady, 1992; Westlin, 1992). These data are consistent with the observation that only newly formed cytoskeleton-associated actin is depolymerised by the addition of cytochalasin D (Cano, 1991).

The time course of actin polymerisation following smoke exposure was not determined. However, polymerisation occurred rapidly as the cells were fixed and stained for F-actin immediately following exposure. The filtration pressures of smoke

exposed neutrophils measured over 6 minutes did not demonstrate a recovery in cell deformability, suggesting a sustained increase in polymerised actin as is the case with PMA stimulated neutrophils (Howard, 1987)

Several studies have reported polymerisation of actin monomers at 37°C compared with room temperature, and depolymerisation when cells were placed at cold temperatures (Cassimeris, 1990; Downey, 1988). However, Aoshiba and associates (1993) measured a decrease in deformability when neutrophils were incubated for 15 minutes at a range of temperatures from 40°C to 4°C. As the effect of temperature on deformability could be reversed by rewarming or by addition of cytochalasin B, their data therefore suggests increasing actin polymerisation as temperatures fall.
Despite the confusion over the effect of temperature on cellular actin status and deformability, it is unlikely that the marked increase in F-actin and reduced deformability measured following smoke exposure was due to a temperature effect. Although an increase in F-actin was measured in this study by prolonged incubation at 37°C (data not shown), 4 minutes at 37°C during smoke exposure was not sufficient to increase the F-actin content of neutrophils as control and sham exposed cells had similar levels. In addition, the deformability of control and sham exposed neutrophils was not different.

Actin polymerisation is associated with a decrease in intracellular pH and an increase in calcium levels (Downey, 1989; Lassing, 1985; Naccache, 1987; Sklar,1985). However, the influence of pH on actin polymerisation is not clear. Athough several authors reported F-actin assembly was not mediated by a decrease in intracellular pH (Downey, 1989; Naccache, 1987), Sampath and Pollard (1991) reported slower actin polymerisation at alkaline pH. They found the dissociation rate of actin monomers at the barbed end to be higher at alkaline pH, and the association rate at the pointed end to be slightly reduced resulting in slower filament growth under alkaline conditions than under acid conditions. Chien and colleagues (1984) found neutrophil deformability was reduced at alkaline pH. This was, however, associated with cell swelling and hence a loss of the excess membrane surface area available for deformation, although rigidity of the cell cytoskeleton was also postulated as a possible mechanism. In a more recent study Aoshiba and co-workers (1993) showed no change in neutrophil deformability over a range of pH (6.8 to 7.6 at 25°C). In the present study the pH of buffer decreased when exposed to smoke (section 2.4.4) but was still within the range Aoshiba et al showed had no

effect on neutrophil deformability. Thus the reduced deformability following smoking is unlikely to be mediated by an effect of pH on actin polymerisation.

The increase in intracellular calcium (Ca^{2+}) concentration which occurs with cell stimulation is not a requirement for actin polymer formation in neutrophils (Howard, 1987; Naccache, 1987; Sklar, 1985). Thus it appears unlikely that the smoke-induced increase in F-actin in neutrophils was due to an increase in intracellular Ca^{2+} levels. However, membrane Ca^{2+} is necessary for signal transduction (Bengtsson, 1990; Smolen, 1982) and intracellular calcium may also have a regulatory role for actin polymerisation/depolymerisation. Several of the actin binding proteins involved in the formation and control of the cytoskeletal network are Ca^{2+} dependent (Bearer, 1993; Janmey, 1987; Lassing, 1985), and depolymerisation of actin is thought to be regulated by the Ca^{2+} concentration (Sklar, 1985). Smoke-induced changes in cellular calcium concentrations may, therefore, play a role in reorganisation of the neutrophil's cytoskeletal network during smoke exposure.

A HETEROGENOUS F-ACTIN RESPONSE TO *IN VITRO* SMOKE EXPOSURE

Two sub-populations of smoke exposed neutrophils were apparent on flow cytometric analysis of cellular F-actin content (Figure 5.10c). A proportion of the cells were, for most subjects, unaffected by smoke exposure, whereas a marked increase in fluorescence intensity was observed for the remainder of the population. A heterogeneous response of the neutrophils to smoke exposure was not evident on filtration of the neutrophil suspensions. However, since the less deformable cells dominate the pressure measurement in the constant flow system (Jones, 1984; Nash, 1988b), the presence of cells with unaltered deformability would be masked. The data for individual cell deformability in chapter 4, using the cell transit analyser (CTA) and micropipette aspiration, also suggest the presence of a subpopulation of cells which were less or unaffected by smoke exposure.

Worthen and associates (1989), measuring cell deformability using their 'cell-poker', found a subpopulation of neutrophils which were unresponsive to fMLP stimulation. Heterogeneity in neutrophil deformability following fMLP stimulation was also observed by Frank (1990a) who measured the transit time of the passage of neutrophils through a single-pore membrane. In addition, Wallace and associates (1984) found up to 30% of fMLP-stimulated neutrophils did not polymerise actin.

Wallace et al (1984) speculated that the heterogenous response to fMLP-induced actin polymerisation may be due to perturbations of the harvesting procedure, or the maturity of the neutrophils. Moreover, a bimodal pattern was found to develop

spontaneously with time when the membrane potential of neutrophils was monitored (Seligman, 1981). Whether the harvesting procedure, cell maturity or time post sampling was the cause of the heterogeneous population following smoke exposure was not investigated. Although the neutrophils actin content was assessed immediately following harvesting, by the nature of the harvesting procedure, the cells were exposed to smoke 2 to 2.5 hours post blood sampling. However, there was no evidence of a bimodal population following sham exposure, where neither the harvesting procedure nor time affected neutrophil actin content. It is still possible that a further insult, such as smoke exposure, may be required to induce population heterogeneity.

REORGANISATION OF INTRACELLULAR F-ACTIN IN NEUTROPHILS BY SMOKE EXPOSURE

As well as different degrees of actin polymerisation, there appear to be distinct patterns of F-actin redistribution within neutrophils in response to different stimuli. Fluorescence staining of actin filaments demonstrated a diffuse distribution of actin throughout the cytoplasm or a thin cortical rim in quiescent neutrophils (Coates, 1992; Howard, 1985; Roos, 1987; Sheterline, 1986; Wallace, 1984; Westlin, 1992). Following stimulation with triggers such as fMLP, IL-8, C_{5a}, and ZAP initially the pattern of F-actin distribution shifted to the subcortical region whilst the neutrophil retained its round shape (Coates, 1992; Howard, 1985; Wallace, 1984; Westlin, 1992). Over the next several minutes actin microfilaments in the neutrophil rearranged asymmetrically into a broad front end (pseudopod) with a smaller rear tail region (uripod), producing the distinct head-tail polarity which is frequently observed (Coates, 1992; Howard, 1985; Wallace, 1984; Westlin, 1992). This shape change remains for up to 15 minutes (Westlin, 1992) whilst the F-actin content of the cell decreases and deformability improves (Frank, 1990a; Pecsvarady, 1992). In contrast, no re-distribution of actin has been observed following PAF or LTB_4 (Westlin, 1992).

Visualisation of F-actin in smoke exposed neutrophils revealed accentuated subcortical fluorescence compared to sham exposed cells with no evidence of head-tail polarity (Figure 5.12). The depolymerisation of F-actin by the subsequent addition of cytochalasin B to smoke exposed neutrophils is evident on the photomicrographs by the presence of only diffuse fluorescence (Figure 5.12).

ACTIN POLYMERISATION AS A MECHANISM FOR IMPAIRED NEUTROPHIL FUNCTIONAL BEHAVIOUR

Cytoskeletal actin was found to inhibit the fMLP-induced respiratory burst oxidase activity when actin was 'clamped' in its polymerised form by phalloidin (Al-Mohanna, 1990). Yuli and associates (1982) suggested fMLP-receptor interaction with filamentous actin resulted in inhibition of O_2^- release, although for other agonists, such as tumour necrosis factor, polymerised actin was required to maintain O_2^- release. Hence, fMLP-induced O_2^- release was enhanced by inhibition of actin polymerisation by addition of the inhibitor cytochalasin B (Yuli, 1982), and is therefore routinely used in assays to enhance the oxidative response to a stimulant (Al-Mohanna, 1990; Curnutter, 1975). The increased subcortical actin observed in smoke exposed neutrophils may explain the diminished oxygen radical release by such cells (Section 4.4.5). Moreover, as F-actin re-distribution to the submembrane of the cell prevents neutrophil adhesion and locomotion (Westlin, 1992), a reduction in adherence and chemotaxis of smoke exposed cells (Bridges, 1977; Rasp, 1978; Selby, 1992) may be due to the increased cortical F-actin following smoke exposure.

THE EFFECT OF SMOKE EXPOSURE ON MICROTUBULES

Colchicine, a specific inhibitor of microtubule formation (Brown, 1979), did not alter the smoke-induced change in neutrophil deformability. As neutrophils were incubated with colchicine prior to smoke exposure, the lack of an effect may have been due to interference of cigarette smoke with colchicine's inhibitory action. However, addition of colchicine post smoke exposure also did not alter the deformability of smoke exposed neutrophils (data not shown). Moreover, pre-incubation with colchicine did not prevent the fMLP-induced reduction in neutrophil deformability. That microtubules do not play a major role in whole cell deformation was also demonstrated by Frank (1990a) for fMLP stimulated neutrophils. Microtubules may have some effect on cell deformation, however, by their influence on intracellular viscosity and elasticity (Chien, 1984).

As microtubules are involved in cytosol organisation such as the movement and exocytosis of lysosomes (Olmsted, 1973), their disruption may explain the reduced proteolytic activity of *in vitro* smoke exposed neutrophils (Brown, 1991). In addition the reduced neutrophil chemotaxis, which occurs following smoke exposure, both *in vivo* (Noble, 1975) and *in vitro* (Bridges, 1977; Selby, 1990), may also be explained by a dysfunction of the microtubules of the cell as they are required for neutrophil chemotactic behaviour (Malech, 1977; Newton, 1989).

5.5.2 CIGARETTE SMOKE CAUSES AN OXIDANT STRESS

Several innate antioxidant defense systems exist *in vivo* such as superoxide dismutase (SOD), catalase, glutathione peroxidase, α-tocopherol, ascorbic acid, beta-carotene, and protein and non-protein sulfhydryls. Antioxidants are believed to neutralise phagocyte-derived reactive oxidants generated during the smoke-induced inflammatory reaction (Anderson, 1974; Hoidal, 1981, Richards, 1990), thereby preventing inactivation of α_1-proteinase inhibitor in the lungs and the consequent tissue destruction by uninhibited leucocyte proteases (Janoff, 1983a). Moreover, antioxidants may protect tissues against the direct oxidative effect of cigarette smoke *in vivo*. Ascorbate, beta-carotene, α-tocopherol and α-$_1$-proteinase inhibitor are reduced in plasma and lungs as a result of smoking (Calder, 1963; Janoff, 1979b; Pacht, 1986; Roberts, 1993; Theron, 1990), which has been associated with abnormal lung function (Janoff, 1983b; Richards, 1989). However, other antioxidants such as glutathione (GSH) (Cantin, North et al., 1987; Toth, Berger et al., 1986), ceruloplasmin (Galdston, 1984), SOD (Hoidal, 1981; McCusker, 1990) and catalase (McCusker, 1990; Toth, 1986) were increased. Oxidation of plasma protein thiols and bilirubin was also noted following *in vitro* smoke exposure, but not α-tocopherol or uric acid (Frei, 1991).

ANTIOXIDANTS PROTECT AGAINST THE SMOKE INDUCED CHANGE IN CELL DEFORMABILITY

In this study, smoke exposed neutrophils suspended in plasma (2 ml) developed lower filtration pressures than cells exposed to smoke when suspended in buffer. Similarly, the antioxidants GSH and albumin, at concentrations present in plasma (3 μM and 3% respectively)(Bridgeman, 1991; Cantin, 1987), reduced the effect of smoke exposure on cell filtration pressures (Figure 5.3). Furthermore, the presence of low concentrations of erythrocytes effectively ameliorated the effect of smoke on neutrophil filtration pressures (Figure 5.3).

That intracellular erythrocyte antioxidants can protect other tissues from oxidant injury has previously been demonstrated (Mangione, 1991; Toth, 1986 & 1984). Likewise, the antioxidant potential of albumin is well established (Halliwell, 1988). It functions as a sacrificial scavenger, thereby preventing inactivation of the essential protein and lipid sulfhydryls. In addition albumin does not itself perpetuate further radical activity (Halliwell, 1989). Glutathione is a prevalent cellular thiol which acts as a reducing agent and an antioxidant. Glutathione is synthesized in the liver and, although the liver is a main user of glutathione, large amounts are exported into plasma and subsequently available in other organs (Deneke, 1989). The lungs,

kidneys and intestinal epithelium are probably the organs which utilise blood GSH most (Deneke, 1989). The majority of cells, with the exception of kidney cells (Hagen, 1988) and possibly lung epithelial cells (Hagen, 1986), do not transport intact GSH into cells. To synthesize their own GSH these cells require uptake of the precursor amino acids glutamine, cysteine and glycine across cell membranes.

Endogenous glutathione and glutathione-utilising enzymes provide a complex defense system against oxidative injury (Meister, 1983a; Meister , 1983b). The glutathione system (Figure 5.13) functions to maintain intracellular glutathione predominantly in the reduced state (GSH)(Deneke, 1989) and to scavenge oxidants. Hydrogen peroxide, for example, is reduced by GSH via glutathione peroxidase to water. The oxidised form of GSH (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH), mediated by the enzyme glutathione reductase. Alternatively GSSG can react with protein sulfhydryls or be transported out of the cell (Meister, 1983). This could potentially impair protein function (DeLucia, 1975) and deplete the total intracellular GSH. The co-substrate $NADP^+$ is returned to its reduced form via the pentose phosphate pathway.

The importance of GSH in protection against oxidant injury has been established under conditions where its concentration has been reduced (Deneke, 1985; Mangione, 1991; Moldeus, 1985). Under experimental conditions this can be achieved by inhibiting the synthesis of GSH, or by direct depletion *in vitro* or in animal models *in vivo*. GSH synthesis can be inhibited with buthionine sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine (Bilzerg, 1991; Meister, 1983b), the enzyme which is the control point in the synthesis of GSH. The rate of depletion depends on the turnover rate of GSH. GSH concentrations can also be reduced directly by complexing GSH with an electrophilic agent such as diethylmaleate (DEM), although a mitochondrial pool of GSH is reported to remain (Meredith, 1982). However, at high levels such electrophilic agents are toxic to the cell (Deneke, 1985). GSH can also be depleted by exposure to oxidant stress, particularly in the presence of a GSH-reductase inhibitor (such as N,N-bis(2-chloroethyl)-N-nitrosourea) which would prevent the reduction of the oxidised form GSSG (DeLucia, 1975). To keep the intracellular GSH and GSSG concentrations constant, GSSG forms mixed disulfides with cellular proteins or is transported out of the cell (Griffith, 1979). As normal plasma levels of GSSG are very low, an increased GSSG level in plasma could be used as a sensitive indicator of oxidant stress (Abdalla, 1990). Plasma sampling could, therefore, be used to assess whole body (multiple organ) assessment of oxidant stress.

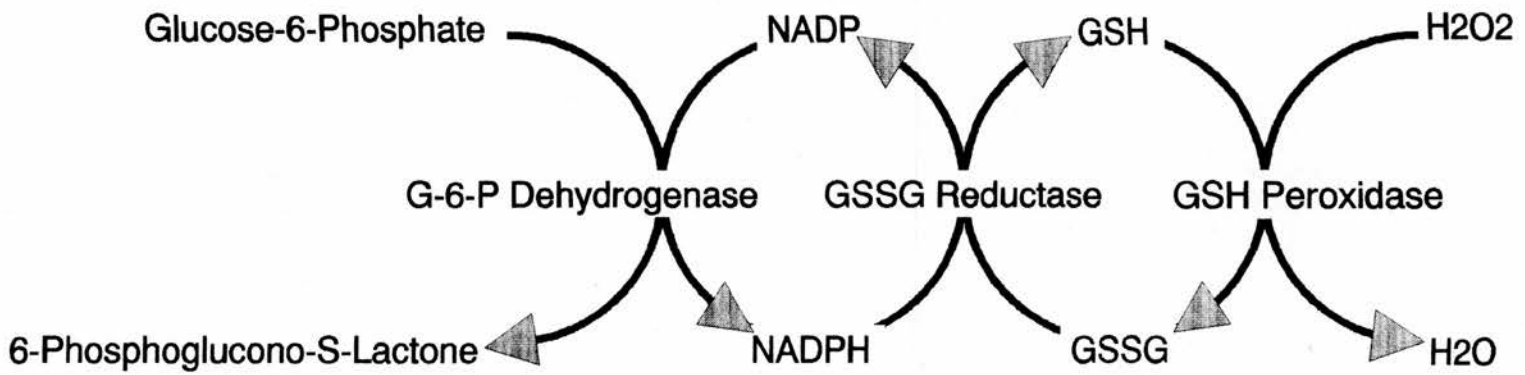


FIGURE 5.13
The glutathione (GSH) system which functions to remove intracellular hydrogen peroxide by its reduction by GSH via glutathione peroxidase. GSH is replenished by reduction of the oxidised GSH (GSSG) by reduced nicotinamide adenine dinucleotide (NADPH).

Moreover, sampling across organs potentially allows assessment of the response of specific organs to oxidant stress. Such data have identified oxidant damage in the lungs following injury to other organs. For example, arterio-venous differences in GSSG across the lungs have been reported following paraquat administration (Adams, 1984) and following intestinal ischaemia (Abdalla, 1990). Total GSH, i.e. the sum of oxidised plus reduced GSH, can also be used to indicate an oxidant stress. Total GSH can be altered by a decrease in GSH excretion from the liver; decreased plasma GSH due to enhanced uptake by organs under stress; and increased levels of GSSG in plasma as a result of translocation out of cells (Deneke, 1989). However, total GSH levels are less likely to be as sensitive an indicator of oxidant stress as the measurement of reduced or oxidised GSH alone.

A striking reduction in intracellular GSH was measured in these studies following exposure of neutrophils to smoke *in vitro* (section 5.4.1). Other workers have found a reduction in GSH levels in lung cells following smoke exposure. Moldeus and associates (1985) measured the depletion of GSH in isolated rat hepatocytes exposed to fresh smoke and cigarette smoke condensate. In an isolated lung preparation, cigarette smoke administration via the trachea diminished total lung GSH which could be protected against by N-acetylcysteine (Moldeus, 1985), a polypeptide reputed to be a precursor of GSH and to have antioxidant activity (Cotgreave, 1986). Likewise, Voisin and associates (1985) found the cytotoxic effect of vapour phase cigarette smoke on guinea pig alveolar macrophages was dependent on the cellular GSH content. Moreover, Younes and Robke (1988) showed inhibition of PMA-stimulated O_2^- and H_2O_2 release by neutrophils by prior depletion of GSH using phorone (diisopropylidene acetone), as found in the present studies following *in vitro* smoke exposure (section 4.4.5). Moreover, reduction of sulfhydryls as a result of oxidant stress was observed to induce actin polymerisation (Hinshaw, 1988; Mirabelli, 1989).

Although a change in neutrophil deformability was prevented when the cells were exposed to smoke when suspended in a 2 ml volume of plasma, exposure in a reduced (1 ml) volume provided less protection (Figure 5.4), presumably as more surface area of the cell suspension was exposed to the cigarette smoke. This has physiological relevance considering that neutrophils within the pulmonary microcirculation are surrounded by very little plasma (Skalak, 1989). However, neutrophil deformability was equally preserved when cells were exposed to smoke in a 1 ml as in a 2 ml volume each containing 3 μM GSH (section 5.4.1). These data

demonstrate the importance of the antioxidant potential of a solution. This is reflected in the increased antioxidant potential measured in blood of smokers compared with non-smokers (Galdston, 1984; McCusker, 1990; Toth, 1986). Moreover, a two-fold higher GSH concentration is present in lung surfactant of smokers compared with non-smokers (Cantin, 1987).

EXPOSURE TO HOCl REDUCES NEUTROPHIL DEFORMABILITY

The presence of various antioxidants protected neutrophils from the detrimental effect on cell deformability during *in vitro* smoke exposure. This suggests the smoke-induced injury is oxidant mediated. Support for this hypothesis comes from the studies with HOCl. Addition of the oxidant HOCl (150 μM) to neutrophils resulted in an increase in cell filtration pressures, i.e. reduced neutrophil deformability (Figure 5.5). HOCl was chosen as it is a major oxidant produced by neutrophils during inflammation (Winterbourn, 1985). It is possible that HOCl could have activated the neutrophil which would also cause an increase in filtration pressures as observed following fMLP and PMA stimulation. However, exposure to both chemical and enzymatically generated oxidants such as HOCl or H_2O_2 has previously been shown to cause several biochemical perturbations suggestive of injury. These include activation of the hexose monophosphate shunt (HMPS) and glutathione redox cycles with loss of GSH (Schraufstatter, 1985); inhibition of ATP synthesis and hence a reduction in cellular ATP levels (Mirabelli, 1989; Spragg, 1985; Timerman, 1990); DNA damage (Schraufstatter, 1986) and disruption of the microfilaments causing gross changes to the plasma membrane (Hinshaw, 1986; Mirabelli, 1988a, 1988b & 1989; Patel, 1988; Timerman,1990). Moreover, although GSH levels were not assessed following exposure to HOCl in this study, Bilzer and colleagues (1991) found a marked depletion of in HOCl exposed neutrophils.

5.5.3 THE EFFECTS OF SMOKE EXPOSURE ON THE NEUTROPHIL PLASMA MEMBRANE

The reduced cell deformability following smoke exposure could also be due to changes in the plasma membrane of the neutrophil. A direct effect of cigarette smoke oxidants, or an enhanced release of oxygen radicals by neutrophils, may peroxidise membrane lipids resulting in molecular and physical reorganisation of the plasma membrane.

PEROXIDATION OF THE PLASMA MEMBRANE LIPIDS

A more severe oxidant stress was required to produce lipid peroxidation than the dose required to alter neutrophil deformability or deplete GSH, which supports data from other workers (Hyslop, 1988; Timerman, 1990). No significant change in MDA levels could be detected under normal exposure conditions, i.e. 2 ml of neutrophil suspension exposed to 5 puffs of vapour phase cigarette smoke over 4 minutes. However, lipid peroxidation was evident, measured by increased TBA reactivity, following a higher dose of smoke (0.5 ml of neutrophil suspension exposed to 5 puffs over 4 minutes). It should be remembered that the TBA test is not specific for MDA. The yield of free MDA from lipid oxidation is reported to be low (Hecker, 1987) and thus may be insufficient to give a positive reaction in the assay. Most of the MDA detected in the assay is formed by decomposition of peroxides when the sample is heated under acidic conditions. This does, however, have the advantage that peroxides which have not decomposed to MDA are still detected in the TBA test. Moreover, several other carbonyl compounds (such as aldehydes and ketones), when heated with MDA, will form products that absorb light at 523 nM. Conversely, free MDA can be rapidly metabolised, particularly *in vivo*, by strong oxidants such as millimolar amounts of H_2O_2 (Kostka 1989). The TBA test, therefore, can only give a crude index of lipid peroxidation. Although there are other end products of lipid peroxidation in addition to MDA which can be measured (Halliwell, 1989), the simplicity of this test is an advantage and for the purpose of this study the TBA test was sufficient. As the assay tends to overestimate the levels of MDA, it strengthens the observation that no peroxidation of neutrophil membranes was evident following exposure to a dose of smoke which had a marked effect on cell deformability.

Despite the inadequacies of the TBA test, this and other assays have demonstrated that lipid peroxidation occurs following smoke exposure *in vitro* and *in vivo*. Nadiger and colleagues (Nadiger, 1987) found serum MDA levels were higher in smokers. Also, Lentz and DiLuzio (1974) reported increased TBA reactive substances from lipids in rabbit alveolar macrophages following exposure *in vitro* to aqueous cigarette smoke. Moreover, Frei and co-workers (Frei, 1991), using a sensitive HPLC technique, detected lipid hydroperoxides in plasma exposed *in vitro* to the gas phase cigarette smoke, but not whole smoke.

CIGARETTE SMOKE EXPOSURE AND MEMBRANE FLUIDITY

Masuda and colleagues (1990) found the membrane fluidity of peripheral blood leucocytes, measured by fluorescence spectroscopy, to be decreased following

stimulation with PMA and high concentrations of fMLP (10^{-5}M). PMA and higher concentrations of fMLP evoke neutrophils to release oxygen radicals which, by peroxidation of the membrane, may decrease the fluidity of the plasma membrane. In support of their hypothesis, Masuda et al (1990) demonstrated that the presence of radical scavengers, particularly catalase (thereby implicating H_2O_2) preserved the membrane fluidity of stimulated neutrophils. Previous workers have shown a reduction in the fluidity of membranes for several cell types as a result of exposure to reactive oxygen intermediates (Dobretsov, 1977; Ingraham, 1981; Rosen, 1983). Moreover, Rietjens and associates (1986) found that rat alveolar macrophages subjected to ozone or nitrogen dioxide in an *in vitro* system had a reduced membrane fluidity which could be protected against by preincubation with antioxidants. Also Patel and Block (1988) have shown that nitrogen dioxide and high concentrations of oxygen can cause changes in membrane phospholipids of cultured pulmonary artery endothelial cells with an associated decrease in fluidity. Cigarette smoke could potentially affect the plasma membrane of cells by a similar mechanism as the smoke from one cigarette contains 10^{16} free radicals (Pryor, 1983) and potent oxidant gases such as nitrogen dioxide (Church, 1985).

As the lung is the primary target organ for cigarette smoke exposure, lung cells and capillary endothelial cells are the critical target cells for oxidant induced changes in the membrane lipids. Hannan and associates (1989) found reduced membrane fluidity in rat alveolar macrophage after 2 weeks exposure to smoke, which persisted up to 18 weeks post exposure. They proposed the changes in macrophage function associated with cigarette smoking, such as decreased migration, adherence, granule/membrane fusion, decreased Fc-receptor affinity, and increased superoxide release which all involve the plasma membrane, may be due to altered membrane fluidity.

Cigarette smoke exposure may alter membrane fluidity by several mechanisms. Oxidant gases and free oxygen radicals may cause changes in membrane phospholipids, by oxidation of double bonds in acyl side chains, with an associated decrease in membrane fluidity. Another possibility is an increase in the cholesterol: phospholipid ratio of the plasma membrane or an increase in the fatty acid content, which would decrease membrane fluidity. Indeed, Latha and associates (1988) reported an increase in cholesterol in the serum and organs of chronic smoke exposed rats.

Moreover, actin polymerisation could also influence the fluidity of the neutrophil plasma membrane. Enhanced polymerisation of actin, which is linked to the membrane by intra-membrane protein linkage, could limit the mobility of membrane lipids (Edelman, 1973; Schlessinger, 1983). A reduction in membrane fluidity was also associated with a decrease in cellular deformability for neonate blood cells (Newton, 1989). The cell membranes of neonatal neutrophils were significantly less fluid than those of adult neutrophils (Newton, 1989), but the fluidity could be enhanced by pentoxifylline, a methylxanthine derivative which was shown to decrease neutrophil F-actin levels (Rao, 1988) and enhance neonatal neutrophil chemotaxis (Hill, 1987). Likewise, a decreased membrane fluidity was found for erythrocytes from neonates and related to increased cell rigidity and impaired deformability (Crespo, 1988). Furthermore, McKay and associates (1991), in a recent study of fMLP-induced polarity in neutrophils, found greater mobility for the lipid probe at the front than at the back of the cells, which they speculated may be due to both redistribution of membrane proteins and cytoplasmic F-actin.

A slight, but insignificant decrease in lateral diffusion of the neutrophil plasma membrane was observed following smoke exposure for both the fluorescent lipid probes used in this study (Figures 5.8a and b). Although the lack of change in membrane fluidity observed for smoke exposed neutrophils in this study would appear to be consistent with the absence of lipid peroxidation following smoke exposure, the marked increase in F-actin could be expected to influence lateral mobility of membrane lipids (Masuda, 1990). Moreover, Hannan and associates (1989) showed smoke exposure had a significant effect on the membrane fluidity of macrophages, and other workers reported changes in the bilayer mobility following exposure to oxidant gases (Patel, 1988). That no significant effect of cigarette smoke on membrane fluidity was measured in the present study may be due to the size of the bleached area on the neutrophil surface. As a large proportion of the cell (approximately 25%) was bleached, greater diffusion of the fluorophores was required for an increase in fluorescence in the bleached area. In support, the maximum recovery of fluorescence in the bleached area (extrapolated to infinite time) was low and not significantly different for sham or smoke exposed cells.
Two different fluorophores were used to measure the mobility of membrane lipids in this study, to assess fluidity of the membrane lipids at different sites. A change in membrane fluidity detected using one probe may be peculiar to that probe and not representative of general lipid mobility. That the two lipid probes gave a similar result, would suggest the data was representative for the fluidity of the outer leaflet

of the membrane, and did not pertain to the particular probe employed (Patel, 1988). The lack of an effect of cigarette smoke exposure on neutrophil membrane fluidity in this study may also be due to the limited number of neutrophils assessed and the large range for the measurements of lateral diffusion obtained in each sample.

In summary, the results presented in this chapter show that the mechanism of reduced neutrophil deformability following *in vitro* smoke exposure is due to an increase in cellular F-actin content and it's redistribution to the subcortical region of the cell. This increase in F-actin appears to be oxidant mediated. The oxidant stress was not, however, associated with peroxidation of the plasma membrane lipids at a dose of smoke which was detrimental to cell deformability, nor did it alter plasma membrane fluidity.

CHAPTER 6
CONFIRMATION OF A CHANGE IN LEUCOCYTE DEFORMABILITY FOLLOWING ACUTE SMOKING IN MAN

6.1 INTRODUCTION

Acute cigarette smoking enhances the normal sequestration of neutrophils within animal (Bosken, 1991), and human (MacNee, 1989d) lungs which is considered to be due to changes in the mechanical properties of the neutrophil, i.e their deformability (Downey, 1988 & 1990; Worthen, 1989). A reduction in neutrophil deformability was demonstrated following *in vitro* cigarette smoke exposure in chapter 4. The aim of this chapter was to assess changes in the deformability of blood leucocytes following acute cigarette smoking *in vivo* .

Since the acute effect of smoking *in vivo* may be lost during the procedures necessary to harvest neutrophils, leucocyte deformability was assessed as a component of whole blood. Blood flow is influenced by temperature, shear forces, plasma viscosity, haematocrit, and erythrocyte aggregation, as well as the deformability of erythrocytes and leucocytes (Lowe, 1987). Although leucocytes contribute little to flow resistance in large vessels, in small vessels the leucocytes, due to their larger volume and more complex structure, have greater influence on blood flow (Schmid-Schonbein, 1980b). The passive biconcave disc shape of the erythrocyte is less resistant to deformation (Skalak, 1969). Moreover, leucocytes can adhere to vascular endothelial cells (Atherton, 1973).

The dominant effect of leucocytes on microvascular flow has been observed by both *in vivo* microscopy (Bagge, 1976) and in model capillaries (Chien, 1983; Schmid-Schonbein, 1980b; Skalak, 1983). Theoretical calculations from *in vitro* filtration studies have shown that, with time, the less deformable leucocytes come to dominate the pressure/time relationship (Chien, 1984; Skalak, 1983) despite their lower concentration in blood. Indeed, variations in the filterability of whole blood is significantly affected by total white cell count (Alderman, 1981). Moreover, whole blood filtration is decreased in several disease states, such as cerebrovascular (Elwan, 1991; Laurient-Roudaut, 1981), and peripheral vascular disease (Alderman, 1981; Evans, 1993; Nash, 1988c) associated with a reduction in leucocyte deformability (Evans, 1993). Thus a decrease in leucocyte deformability during *in vivo* smoke exposure should be detectable by a reduction in whole blood filterability.

Cigarette smoke contains of the order of 10^{16} oxidant molecules per cigarette (Pryor, 1983). However, in order to affect cells in transit in the pulmonary capillaries, inhaled oxidants in cigarette smoke must penetrate the alveolar capillary membrane. Support for this hypothesis comes from data showing changes in the antioxidant levels in the blood of smokers compared with non-smokers (Calder, 1963; Galdston, 1984;

McCusker, 1990; Pacht, 1986; Toth, 1986) implying the presence of a systemic oxidant burden. Hence, the second aim of the studies in this chapter was to assess whether evidence of an intravascular oxidant stress could be measured in blood following smoke exposure. As an indicator of oxidant damage, levels of lipid peroxidation and the membrane fragility of erythrocytes was assessed. Also, as the depletion of reduced glutathione (GSH) is believed to be a sensitive measure of oxidant stress (Ji, 1991; Pryor, 1991; Smith, 1991), GSH levels following *in vitro* smoke exposure of venous blood and following *in vivo* smoking in venous and arterial blood samples were measured.

6.2 AIMS

To measure any change in leucocyte deformability following *in vitro* and *in vivo* smoke exposure of whole blood.

To determine if an oxidant stress is present in whole blood following smoke exposure *in vivo*, or in samples exposed to smoke *in vitro*.

6.3 METHODS

Whole blood samples were anticoagulated with lithium heparin, except for the whole blood filtration studies and a differential cell count, where edetic acid (EDTA) was used. Informed consent was obtained from all subjects.

6.3.1 *IN VIVO* AND *IN VITRO* SMOKE EXPOSURE

IN VIVO SMOKING

Smoking volunteers were requested to refrain from smoking for 12 hours prior to the study. Subjects were asked to smoke standard medium tar cigarettes (WD & HO Wills, Imperial Tobacco Ltd., Bristol, UK), inhaling each puff.

For the venous blood studies, blood was sampled by Dr's C Selby and K Skwarski from an indwelling peripheral venous cannula (20G: Venflon 2, Helsingburg, Sweden) in 8 healthy smokers (8M: age range 18-70 years) immediately before, and while smoking 1, 2 or 4 cigarettes in succession.

In the arterial blood studies, samples were withdrawn through an indwelling brachial arterial line (18G: Vigon Leadercath, Ecouen, France) in a further 8 healthy smokers (5M: age range 46 - 82 years) immediately before, whilst smoking 1 or 2 cigarettes, and 10 min post-smoking. The subjects in the arterial blood studies were all smokers with variable degrees of airflow limitation (% predicted FEV_1 50.1 ± 23.8%), and were studied when clinically stable.

The percentage carboxyhaemoglobin (COHb) was measured, using a Co-oximeter 282 (Co-oximeter Instrumentation Laboratory, Lexington, MA, USA), and a differential cell count was performed in each blood sample by the department of Haematology, Royal Infirmary, Edinburgh.

IN VITRO SMOKE EXPOSURE

Samples of whole blood (2 ml) obtained from healthy non-smokers, or isolated erythrocytes were exposed either to air or to the vapour phase of 5 puffs of cigarette smoke whilst being gently rotated in a tonometer system at 37°C, as previously described (Section 2.3.4).

The percentage COHb was measured in whole blood samples as an indication of the dose of smoke exposure.

6.3.2 WHOLE BLOOD FILTRATION

The filterability of whole blood was measured using the constant flow filtration system and Nuclepore membranes with 5 μm (± 0.05 μm) diameter pores as described in section 2.3.3. Samples of blood were diluted to 4% by volume with phosphate buffered saline (PBS) to give approximately 1×10^5 PMN.ml^{-1}, the concentration used in previous filtration studies using isolated neutrophils. Each sample was filtered for 6 minutes at a constant flow rate (1.5 ml.min^{-1}).

6.3.3 FILTRATION OF ERYTHROCYTE SUSPENSIONS

Leucocyte depleted erythrocyte suspensions were prepared by washing twice with PBS, removing the plasma buffy coat and resuspension in a 1:1 dilution with either PBS or autologous plasma to give a 50% haematocrit, similar to that in whole blood (Smith, 1978). Erythrocyte suspensions were filtered immediately following sham or smoke exposure as described for whole blood filtration.

6.3.4 ASSESSMENT OF AN OXIDANT STRESS IN BLOOD FOLLOWING EITHER *IN VITRO* OR *IN VIVO* SMOKE EXPOSURE

GSH LEVELS

Levels of reduced glutathione (GSH) were determined in whole blood samples following either *in vitro* or *in vivo* smoke exposure using a high performance liquid chromatographic technique (Cotgreave, 1986b) as described in Section 5.3.1.

ERYTHROCYTE (RBC) FRAGILITY

RBC fragility was assessed by adding 30 μl aliquots of whole blood to 3 ml volumes of a range of sodium chloride concentrations, between 0.0% - 0.85%. The samples were mixed and left at ambient temperature for 30 minutes. The RBC's were pelleted by centrifugation, and the amount of haemolysis measured as the absorbance of supernatant fluid at 540 nm (Baker, 1962). A sigmoid 'fragility curve' was fitted to the data from which the concentration of saline causing 50% lysis could be determined.

LIPID PEROXIDATION

Lipid peroxidation was assessed in plasma and RBC of arterial blood following smoking *in vivo* by measuring the malondialdehyde (MDA) concentrations as detailed previously (section 5.3.2)(Mengel, 1966).

Lipid peroxidation was assessed following *in vitro* smoke exposure of venous blood (1 ml) by measuring the MDA concentrations in plasma and RBC, and also the presence of the diene-conjugated isomer of linoleic acid (octa-9,11-dienoic acid) in plasma by the method of Iversen and colleagues (Iversen, 1985). Following smoke exposure *in vitro* or *in vivo*, blood samples were centrifuged at 1100 rpm for 10 minutes to separate the erythrocytes and plasma. The buffy coat was discarded. The assessment of diene-conjugates of linoleic acid was performed by Mr R Dawkes, Department of Medicine, Royal Infirmary, Edinburgh. In brief, enzymatic hydrolysis was performed by adding plasma to a solution comprising 0.1M Tris (pH 8.9), 1M methanol and 5U.ml^{-1} phospholipase A_2 for 15 minutes at 25°C. Following which 2 ml methanol containing 0.5% acetic acid and 50 μg.ml^{-1} beta-eleostearic acid was added to precipitate the protein and add internal standard. Following centrifugation the supernatant was partially purified on a reverse-phase column by elution with propan-2-ol/acetonitrile and the conjugated dienes and non-conjugated fatty acids (linoleic, linolenic and arachidonic acids) measured using High Powered Liquid Chromatography (HPLC).

6.3.5 STATISTICAL ANALYSIS

The statistical analysis applied to the data in this chapter were performed as detailed in chapter 2. However, a three factor analysis of variance (ANOVA) was performed on the *in vivo* data using a general linear model, Minitab analysis program was used. The factors were subjects (1-8), filtration (1-6 min) and numbers of cigarette smoked (1, 2 or 4); the response variable was filtration pressure in cm H_2O. From the analysis of variance table the variance ratio (F) test was used to identify those factors that significantly affected the response variable. For each factor the mean square for interaction with patient was used as an estimate of error. Measurement of association between pairs of variable was performed by Pearson rank correlations (Snedecor, 1974).

6.4 RESULTS

The COHb measured in both arterial and venous blood samples following *in vivo* smoking increased significantly in a dose dependent manner compared with pre-smoking levels (Table 6.1), confirming that the subjects had inhaled the cigarette smoke. Blood exposed to smoke *in vitro* also showed a significant increase in COHb compared with the sham exposed samples (sham 3.7 ± 1.6%; smoke exposed 10.1 ± 2.6%: n= 21, $p< 0.001$).

6.4.1 WHOLE BLOOD FILTRATION

IN VITRO SMOKE EXPOSURE

Exposure of whole blood *in vitro* to 5 puffs of vapour phase cigarette smoke did not alter the mean filtration pressures significantly (Figure 6.1). There was no significant correlation between the change in P_6 and the change in COHb after smoke exposure (n=20, r=0.311; $p>0.05$).

IN VIVO SMOKE EXPOSURE

Following smoking *in vivo*, filtration pressures in venous blood were unaltered, following one (mean values: P_6 pre 3.0 ± 1.5, post 2.7 ± 1.1 cmH_2O; n=12,$p>0.05$), two or four cigarettes (Figure 6.2). A multiple comparisons analysis showed that the number of cigarettes smoked by the subjects did not have a significant influence on the filtration pressures of venous blood (n=8, $p>0.05$). There was, however, a significant interaction between subjects and the changes in deformability during smoking ($p<0.01$), thus demonstrating a significant variability in the effect of smoking between individuals.

A similar analysis of the filtration pressures of arterial blood showed a significant increase in filtration pressures following 2 cigarettes (n=8, $p<0.05$)(Figure 6.3). There were significant differences in baseline filtration pressure between subjects ($p<0.001$) and, as in venous blood, there was a significant interaction between subjects for the effects of cigarette smoking on filtration pressures ($p<0.01$). These data indicate that the increase in filtration pressures in arterial blood in response to smoking varied between individual subjects. These results in smokers are in marked contrast to those in non-smokers where filtration pressures on 3 consecutive venous blood samples were not significantly different (Figure 6.4).

Filtration pressures following *in vivo* smoking in arterial blood did not correlate with changes in total white cell (arterial r=0.38, $p>0.05$; venous 0.43, $p>0.05$), neutrophil

(arterial r=0.42, p>0.05; venous r=0.25, p>0.05), mononuclear cell concentration (arterial r=0.20, p>0.05: venous r=0.62, p>0.05)(Table 6.2), nor with the erythrocyte haematocrit (pre smoking 0.456 ± 0.03%, following 1 cigarette 0.460 ± 0.03%, 2 cigarettes 0.458 ± 0.03%, post smoking 0.458 ± 0.02%: n=9, r=0.37, p>0.05).
However, a significant correlation was observed between the change in filtration pressure from baseline to 10 minutes after smoking and the change in neutrophil concentration in peripheral blood over the same period (r=0.68, p=0.03).

6.4.2 ERYTHROCYTE FILTERABILITY

The filterability of erythrocytes smoke exposed *in vitro* whilst suspended in either PBS or autologous plasma (PPP) was not different from sham exposed cells (Figures 6.5a and b).

6.4.3 ASSESSMENT OF AN OXIDANT STRESS IN SMOKE EXPOSED BLOOD

GLUTATHIONE LEVELS

The GSH levels in venous blood samples exposed to cigarette smoke *in vitro* were significantly lower than the levels measured in sham exposed samples (sham 1.26 ± 0.15 mM; smoke exposed 1.08 ± 0.14 mM: n=12, p<0.02). However, the mean GSH levels measured in venous or arterial blood were unaltered following smoking *in vivo* (Table 6.3).

RBC MEMBRANE FRAGILITY

RBC fragility was unaltered in venous blood samples following either *in vitro* smoke exposure (%NaCl at 50% lysis, sham 0.42 ± 0.01 %, smoke exposed 0.42 ± 0.01%, n=5, p>0.05) or exposure *in vivo* to two (pre 0.43 ± 0.03% ± %, post 0.43± 0.02% n=13, p>0.05) or four (pre 0.42 ± 0.01%, post 0.41 ± 0.01%; n=8, p>0.05) cigarettes. Similarly, RBC fragility in the arterial blood samples was unchanged when 2 cigarettes were smoked *in vivo* (pre 0.41 ± 0.04%, 2 cigarettes 0.43 ± 0.02%; n=8, p>0.05).

LIPID PEROXIDATION

Lipid peroxidation could be detected, by measuring MDA levels, in plasma (Figure 6.6), but not for RBC (sham 0.52 ± 0.24 nmol $MDA.ml^{-1}$; smoke exposed 0.55 ± 0.24 nmol $MDA.ml^{-1}$: n=8, p<0.05) following smoke exposure of venous blood *in vitro*. Exposure of venous blood samples (1 ml) to a greater dose of cigarette smoke (10

puffs) induced lipid peroxidation in both plasma (sham exposed 0.99 ± 0.2 nmol MDA.ml^{-1}, smoke exposed 2.79 ± 0.9 nmol MDA.ml^{-1}; n=4, $p<0.01$) and RBC (sham exposed 0.22 ± 0.1 nmol MDA.ml^{-1}, smoke exposed 0.54 ± 0.4 nmol MDA.ml^{-1}; n=4, $p<0.05$).

An increase in the percentage of the conjugated dienes of linoleic acid was detected in plasma in 4 of the 5 samples following smoke exposure *in vitro*, although the change in the mean value was not statistically significant (sham 1.41 ± 0.82%, smoke exposed 1.78 ± 0.71%; n = 5, $p>0.05$).

Acute smoking *in vivo* did not significantly increase products of lipid peroxidation in arterial blood as assessed by the levels of MDA (plasma pre 2.8 ± 3.2 nmol MDA.ml^{-1}, 2 cigarettes 2.6 ± 2.9 nmol MDA.ml^{-1}, n=5, $p>0.05$: RBC pre 1.3 ± 1.2 nmol MDA.ml^{-1}, 2 cigarettes 1.6 ± 1.4 nmol MDA.ml^{-1}; n=5, $p>0.05$).

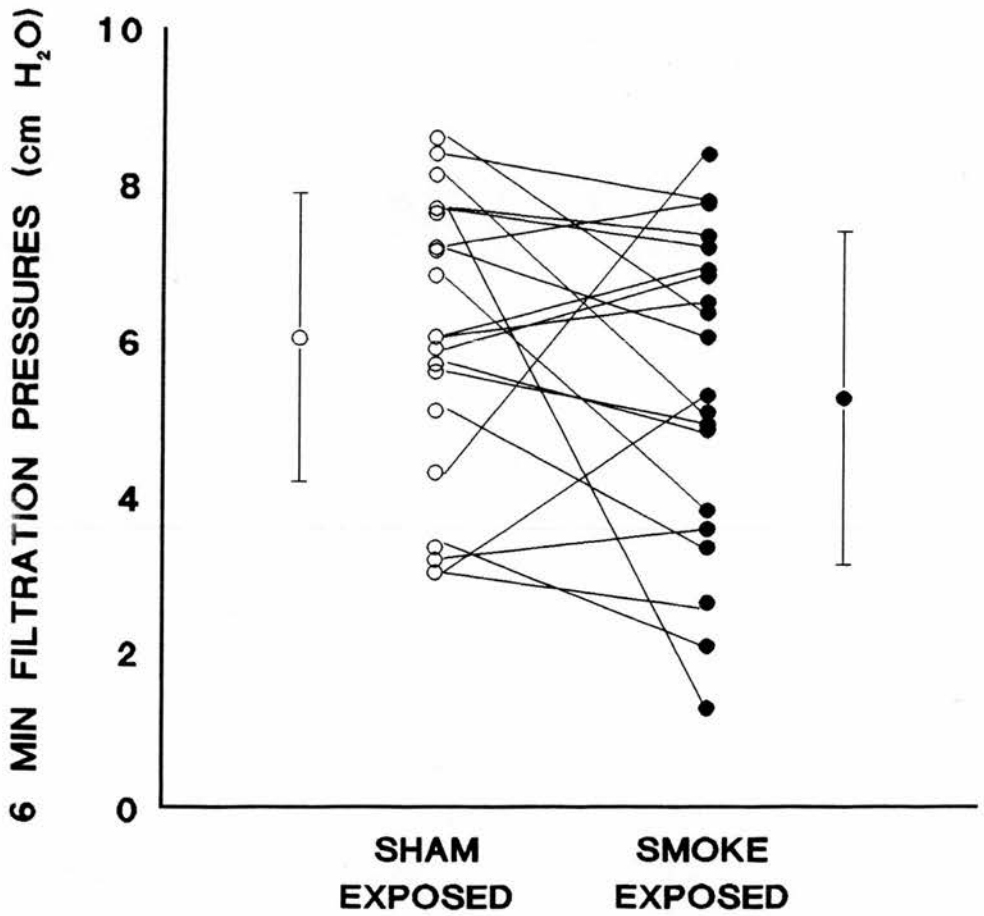


FIGURE 6.1
Plateau pressures developed by venous blood filtered for 6 minutes (P_6) following exposure to smoke *in vitro* (●) compared to samples sham exposed in air (O). *In vitro* smoke exposure did not significantly alter whole blood filtration pressures (n=20, $p>0.05$) (%COHb: sham 3.6 ± 1.6%; smoke exposed 9.7 ± 2.2%: n=20, $p<0.001$). Individual and mean values are shown, error bars indicate 1 SD.

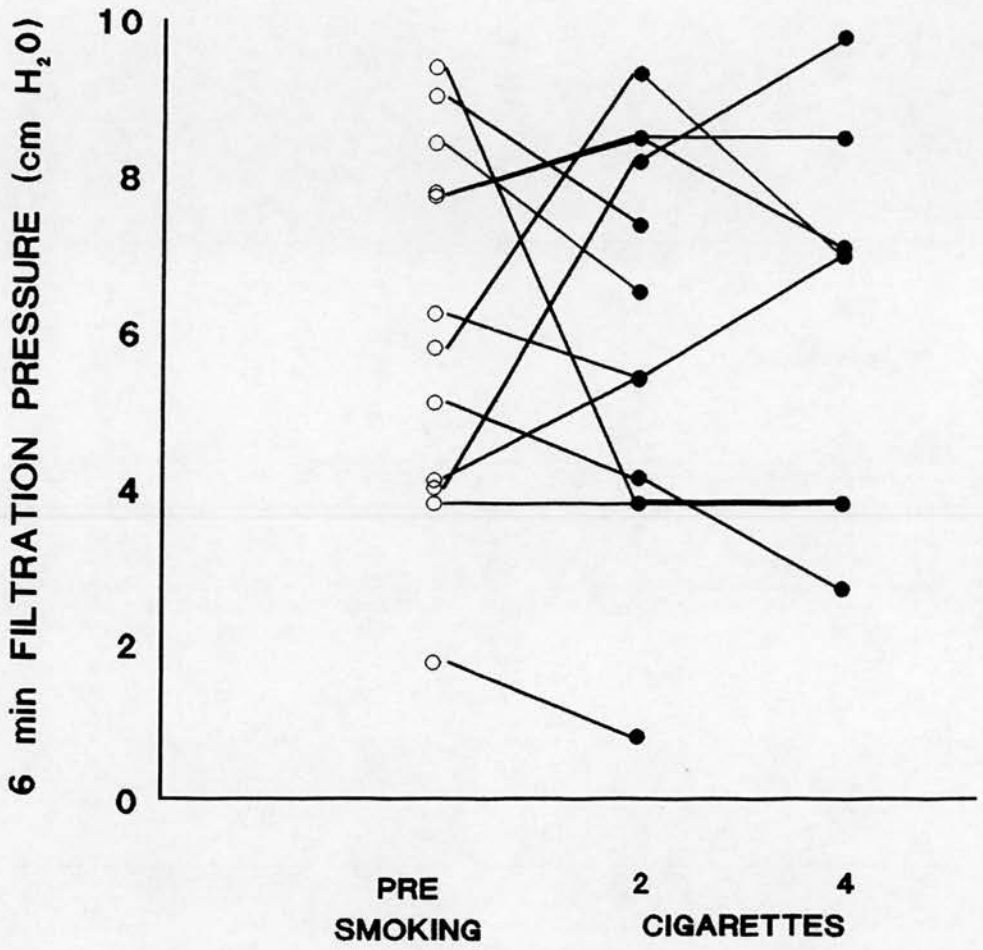


FIGURE 6.2
The pressures developed by 6 minutes filtration (P_6) of venous blood sampled immediately following 2 (n=12) or 4 (n=8) cigarettes (●) smoked in succession. Statistical analysis revealed the number of cigarettes smoked was not a significant factor in influencing the filtration pressures ($p>0.05$), but a significant individual variability in response to smoking was demonstrated ($p<0.01$) (%COHb: pre 3.7 ± 1.7%, 2 cigarettes 6.4 ± 2.2%, 4 cigarettes 8.4 ± 3.2%: $p<0.001$).

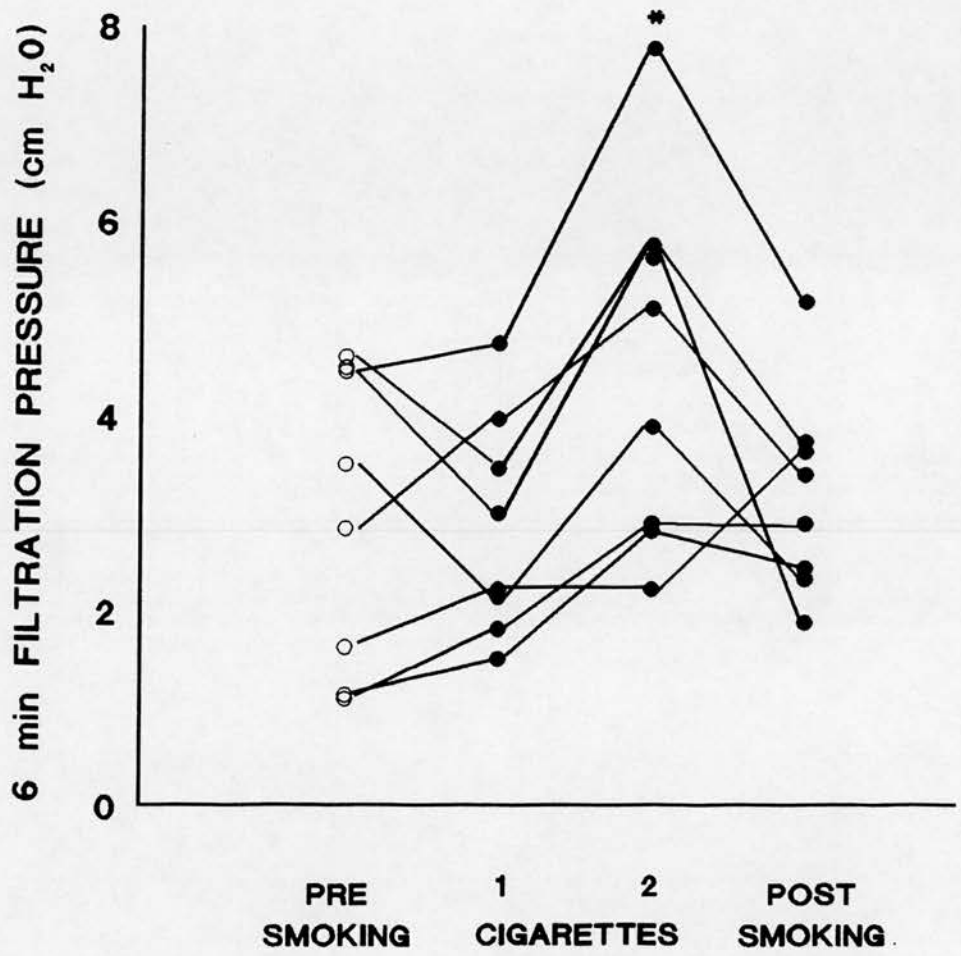


FIGURE 6.3
The pressures developed by the immediate filtration of arterial blood sampled prior to (O), following 1 and 2 cigarettes, and 10 minutes post-smoking (●). An increase in the 6 minute filtration pressure (P_6) was observed in all 8 subjects after 2 cigarettes. Multiple ANOVA showed a significant effect of smoking ($p<0.05$) (%COHb: pre 4.1 ± 1.5%, 1 cigarettes 5.0 ± 1.8%, 2 cigarettes 5.6 ± 2.2%, post 6.0 ± 2.0%; n=8, $p<0.05$).

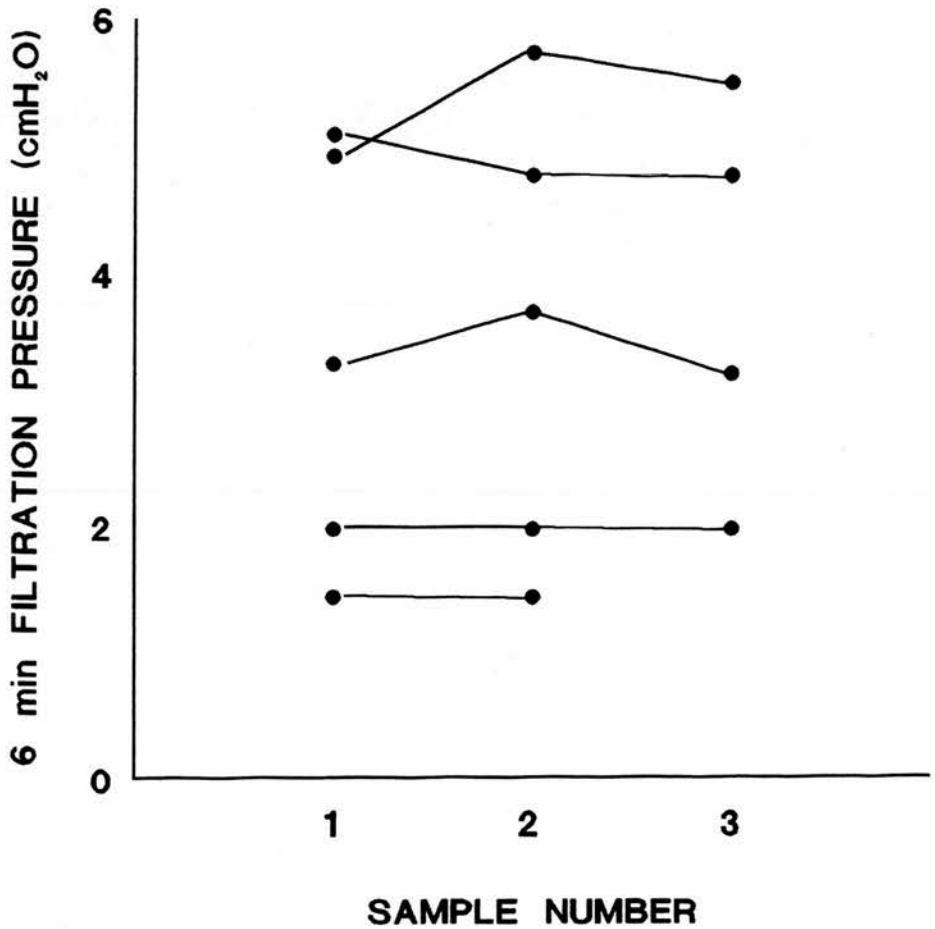


FIGURE 6.4
Reproducibility of whole blood filtration. The results are expressed as the plateau pressures developed by the filtration of diluted whole blood (4% in PBS) over 6 minutes (P_6) sampled from 5 subjects and filtered on two or three separate occasions. Analysis of variance showed no significant difference between consecutive samples ($p>0.25$).

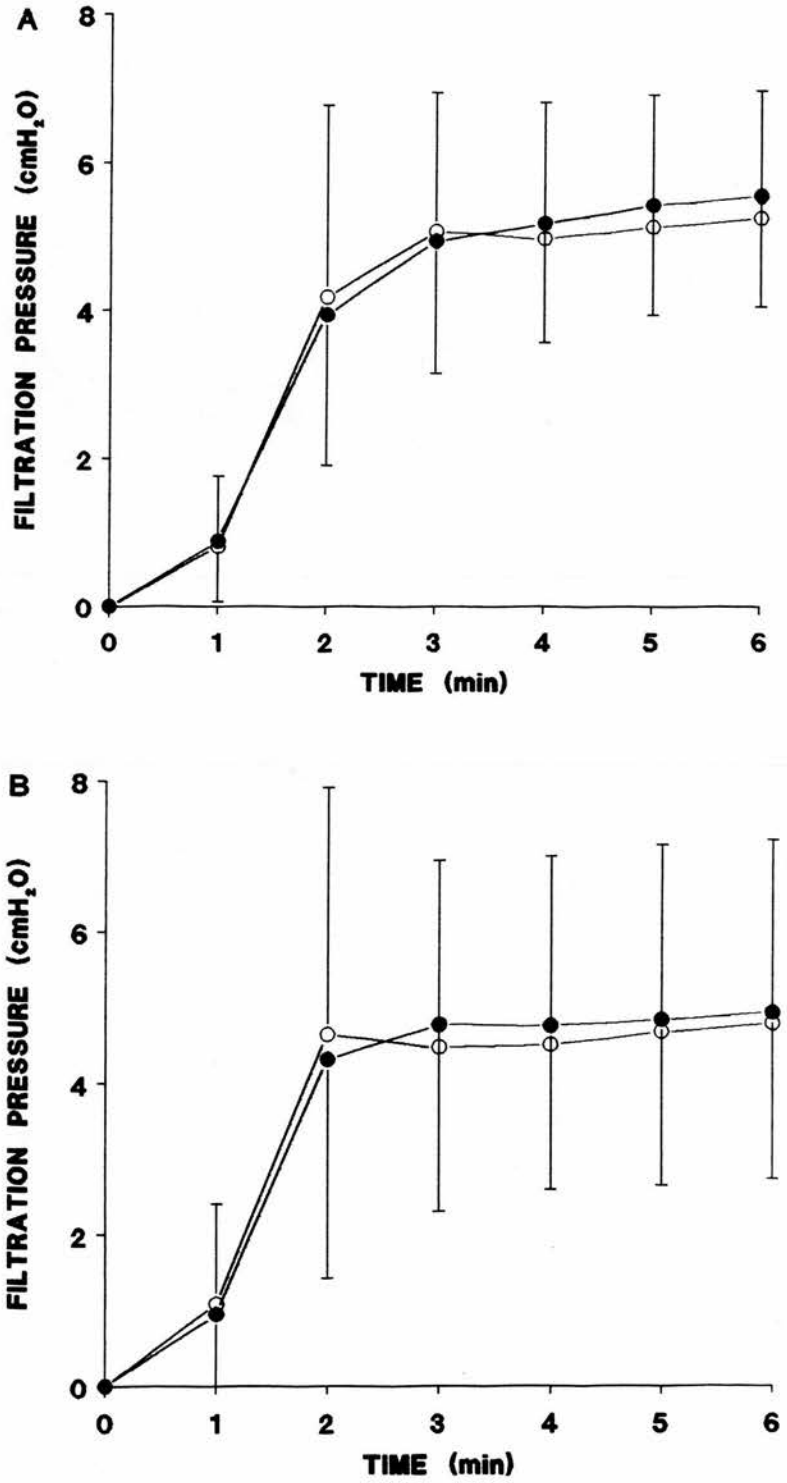


FIGURE 6.5
Filtration pressures developed by sham (O) and smoke exposed (●) erythrocytes suspended in either (a) PBS or (b) autologous plasma. cigarette smoke exposure *in vitro* did not alter erythrocyte filterability through 5 μm pore membranes. Mean values for 9 experiments are shown with error bars representing 1 SD.

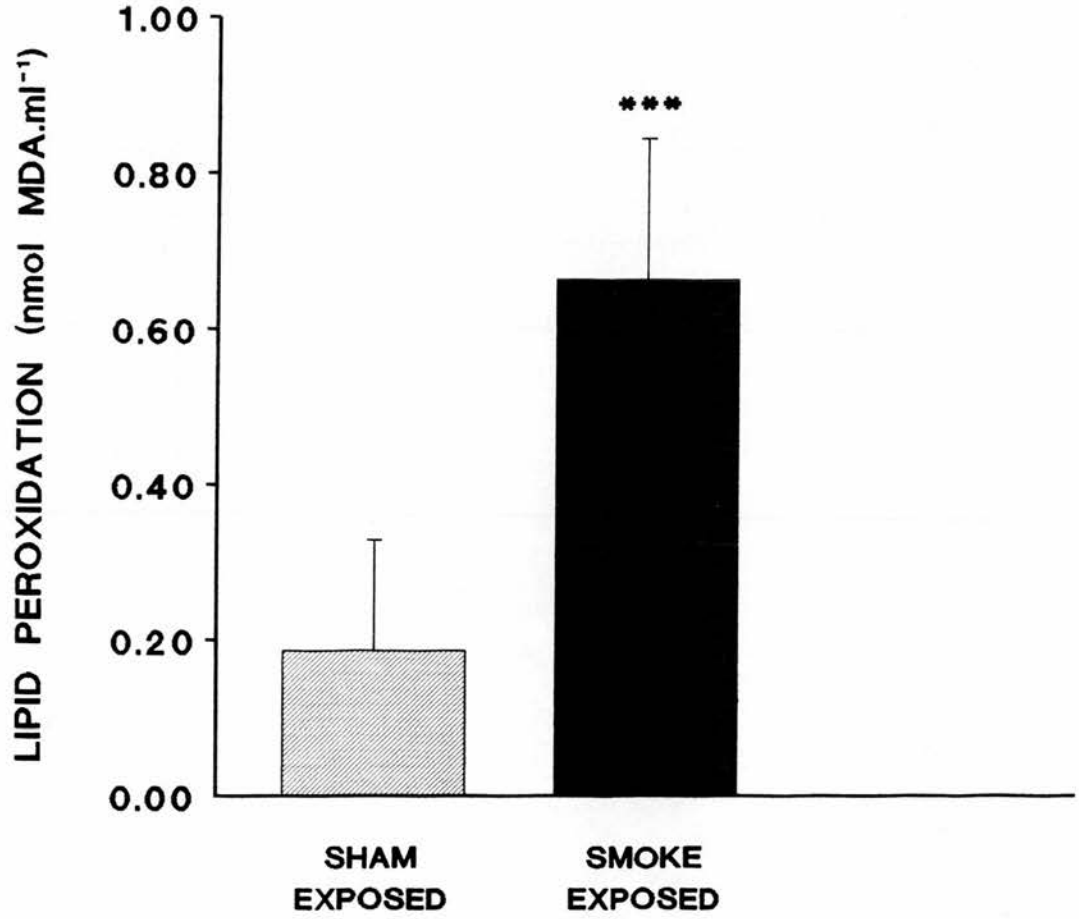


FIGURE 6.6
Plasma levels of malondialdehyde (MDA), as a measure of lipid peroxidation, following *in vitro* smoke exposure of venous blood samples. A marked increase in MDA levels occurred following smoke exposure compared with sham exposed samples (n=6, $p<0.001$). The error bars represent 1 SD.

TABLE 6.1

CARBOXYHAEMOGLOBIN LEVELS (%COHb) IN VENOUS AND ARTERIAL BLOOD FOLLOWING *IN VIVO* SMOKING

		MEAN(SD)				
	n	PRE-SMOKING	1 CIGARETTE	2 CIGARETTES	4 CIGARETTES	POST-SMOKING
VENOUS BLOOD	12	2.9 (1.5)	4.0 (1.6)***	6.4 (2.4)***	-	-
	8	3.7 (1.7)	-	6.5 (2.6)***	8.4 (3.2)***	-
ARTERIAL BLOOD	8	4.1 (1.5)	5.0 (1.8)***	5.6 (1.2)***	-	6.0 (2.0)**

compared with pre-smoking; ** $p<0.005$, *** $p<0.001$

TABLE 6.2

SIX MINUTE FILTRATION PRESSURES (P_6), TOTAL WHITE BLOOD COUNT (WBC), POLYMORPHONUCLEAR LEUCOCYTE (PMNL) AND MONOCYTE (MN) COUNTS MEASURED IN VENOUS AND ARTERIAL BLOOD SAMPLED FOLLOWING ACUTE SMOKING

	MEAN (SD)					
	VENOUS BLOOD		ARTERIAL BLOOD			
	PRE-SMOKING	4 CIGARETTES	PRE-SMOKING	1 CIGARETTE	2 CIGARETTES	POST-SMOKING
P_6 (cm H_2O)	5.40 (2.1)	6.21 (2.4)	2.97 (1.4)	2.86 (1.1)	4.5 (1.8)***	3.2 (1.0)
TOTAL WBC ($10^9.L^{-1}$)	8.75 (1.9)	8.8 (1.9)	6.93 (3.2)	7.65 (2.0)	7.75 (2.1)	7.18 (3.4)
PMNL COUNT ($10^9.L^{-1}$)	5.56 (1.7)	5.74 (1.5)	4.49 (2.5)	4.89 (1.5)	5.21 (2.1)	4.83 (2.9)
MN COUNT ($10^9.L^{-1}$)	0.46 (0.2)	0.42 (0.3)	0.59 (0.2)	0.54 (0.2)	0.56 (0.3)	0.44 (0.1)

Compared with pre-smoking values ** $p<0.001$.

TABLE 6.3

GLUTATHIONE LEVELS (mM) IN VENOUS AND ARTERIAL BLOOD FOLLOWING ACUTE SMOKING *IN VIVO*

		MEAN (SD)				
	n	PRE-SMOKING	1 CIGARETTE	2 CIGARETTES	4 CIGARETTES	CHANGE IN %COHb
VENOUS BLOOD	13	1.36 (0.2)	-	1.33 (0.3)	-	2.4 (0.7)
	8	1.43 (0.2)	-	-	1.4 (0.3)	4.7 (1.7)
ARTERIAL BLOOD	7	1.75 (0.5)	1.75 (0.5)	1.69 (0.4)	-	1.08 (0.5)

6.5 DISCUSSION

Enhanced neutrophil sequestration has been demonstrated in the lungs of healthy smokers during acute cigarette smoking (MacNee, 1989d). Animal studies have shown that the pulmonary capillary bed is the site of this sequestration (Bosken, 1991), as distinct from the neutrophil margination which occurs within the post-capillary venules in the systemic circulation (Schmid-Schonbein, 1980b). As the average diameter of the pulmonary capillaries is smaller than that of the leucocytes, a proportion of these cells have to deform substantially in order to pass through the pulmonary microcirculation. This has major implications in the lungs as the pulmonary capillaries are the site where blood, and hence its cellular constituents, comes in close contact with alveolar air to enable gas transfer. Hence the central hypothesis of this thesis that the increased neutrophil sequestration which occurred during smoking was due to an effect of cigarette smoke on neutrophil deformability.

In chapter 4 a decrease in the deformability of isolated neutrophils was demonstrated following *in vitro* cigarette smoke exposure. Furthermore, neutrophil deformability, measured *in vitro*, correlated strongly with neutrophil sequestration in the lungs *in vivo* (chapter 3). To corroborate these studies, the aim of this chapter was to demonstrate a change in the deformability of intravascular neutrophils during acute smoking *in vivo*. This is particularly important since plasma and RBC have been shown to be protective against smoke induced changes in cell deformability (section 5.4.1) and circulating neutrophils are a component of whole blood.

MacNee et al (1989b) showed that the increase in neutrophil sequestration in the lungs of smokers occurred only during acute smoking. This was subsequently confirmed in animal studies by Bosken and colleagues (1991) who also localised the increased sequestration to the upper lung zones, the preferential site of centrilobular emphysema in cigarette smokers (Thurlbeck, 1963). Also an acute effect of smoking on neutrophil function was demonstrated by Noble and co-workers (1975) who observed a significant reduction in neutrophil chemotaxis after healthy volunteers smoked 2 cigarettes, when compared with smokers who abstained. Similarly, Corberand and colleagues (1979) showed a reduction in neutrophil random motion after subjects smoked 5 cigarettes consecutively. Furthermore, they noted that this did not occur in 4 subjects who did not inhale the cigarette smoke. Acute passive smoking was also found to increase neutrophil numbers, chemotaxis and release of reactive oxygen intermediates of triggered cells (Anderson, 1991).

6.5.1 THE EFFECT OF ACUTE SMOKING ON LEUCOCYTE DEFORMABILITY

The leucocytes in blood, although fewer in number, appear to have greater influence on blood filtration due to their greater cellular viscosity than that of erythrocytes (Schmid-schonbein, 1981; Cokelet, 1968), as determined by theoretical calculations from *in vitro* filtration studies (Chien, 1984; Skalak, 1983). Hence, whole blood filterability measured over time in this study was interpreted as leucocyte filterability, to reflect leucocyte deformability.

In contrast to a change in venous blood neutrophils observed by other workers, in this study mean leucocyte deformability, measured as the filterability of venous blood, was not significantly altered by acute smoke exposure to either 1 cigarette *in vitro* (Figure 6.1) or exposure to 2 or 4 cigarettes *in vivo* (Figure 6.2). However, the effect of smoking on blood filterability was more variable between subjects, compared with control values repeated in the same individual on two or three separate occasions (Figure 6.4). Moreover, a consistent reduction in leucocyte deformability was observed in arterial blood samples after subjects smoked 2 cigarettes, which returned to pre-smoking levels by 10 minutes (Figure 6.3), supporting the central hypothesis that reduced leucocyte deformability occurs during smoking *in vivo*. The lack of any consistent change in filterability in venous blood may be a reflection of a dilution or a recovery effect as venous, as distinct from arterial blood samples, are sampled further from the site of action of smoke in the alveoli on cells in the pulmonary microcirculation.

One difference between the present study and those of Noble (1975) and Corberand (1979), where venous blood leucocytes were affected by cigarette smoke inhalation, is that the blood in this study was anticoagulated with EDTA in contrast to the use of heparin in these previous studies (Corberand, 1979; Noble, 1975). EDTA is a chelator of calcium and could have diminished the effects of smoke exposure, as calcium is required to allow cell activation (English, 1992; Nash, 1988a). However, the arterial blood samples which showed a reduced deformability following smoke exposure were also EDTA anticoagulated. Heparin was not used for these studies because of the possibility of platelet aggregation (Lowe, 1987), and the more rapid activation observed for neutrophils in heparin than cells in citrate or EDTA (Evans, 1984; Plow, 1982). Moreover, the anticoagulant EDTA appears to produce least impairment to the flow of a leucocyte suspension *in vitro* (Nash, 1988a).

A leucocyte-concentration dependent change in filtration pressure has been demonstrated for both pure neutrophil suspensions (section 2.4.3)(Lennie, 1987) and

mixed erythrocyte and leucocyte suspensions (Chien, 1983). However, the increase in filtration pressures in arterial blood samples cannot be explained by a relative increase in the total white cell, neutrophil, or monocyte count (Table 6.2). Interestingly, although the change in total white cell count in arterial blood during smoking did not correlate significantly with the change in filtration pressure, this relationship was significant 10 minutes after cessation of smoking, perhaps reflecting the release of sequestered leucocytes from the lungs. Indeed cigarette smoking has been shown to correlate with the increase in white cell count in peripheral blood (Friedman, 1974). An increase in adrenaline, induced by the nicotine intake from cigarettes (Armitage, 1965), could mobilise the sequestered pool of neutrophils into the circulation, as reported by Muir (1984).

A recent study demonstrated a decrease in the filterability of whole blood in patients with peripheral vascular disease which was due to a reduction in leucocyte filterability (Evans, 1993), although this could not be attributed to the proportion of neutrophils. However, *in vitro* cigarette smoke exposure affected the deformability of isolated mononuclear cells in a similar manner to neutrophils (MacNee, 1989b).

Neither can the increase in filtration pressures be explained by an increase in cell size as morphometric measurement of *in vitro* smoke exposed neutrophils did not show an increase in cell size (section 4.4.5)(Lannan, 1992), and changes in size have little effect on neutrophil filterability (Nash, 1988a).

6.5.2 ERYTHROCYTE DEFORMABILITY FOLLOWING *IN VITRO* SMOKE EXPOSURE

Erythrocytes are particularly sensitive to changes in buffer pH and osmolality, altering the cell volume and thereby affecting the filtration measurement (Reinhart, 1985). Also washing with buffer removes surface proteins and membrane lipids from erythrocytes (Lovelock, 1955). Hence the effect of smoke exposure on erythrocyte deformability was assessed for cells resuspended in plasma. However, as the high antioxidant potential of plasma may reduce or even abolish any effect of smoke exposure, erythrocyte deformability was also assessed for cells in buffer.

Erythrocyte deformability was unaltered by smoke exposure *in vitro* whether the cells were suspended in plasma or PBS (Figure 6.5). However, reduced erythrocyte filterability during smoking has been reported in the literature (Ernst, 1987; Lagrue, 1979; Landgraf, 1981; Norton, 1981), although significance was only attained for subjects who smoked more than 1 pack of cigarettes per day (Ernst, 1987; Norton, 1981). Lagrue and co-workers (1979) also found a reduction in erythrocyte filtration,

measured by whole blood filtration, which correlated with number of cigarettes smoked per day. Norton and Rand (1981) suggested the decrease in erythrocyte deformability in smokers was not as a result of an increased size (absolute volume) or internal viscosity of the erythrocyte, but was due to a reduced cell surface area/volume ratio and/or cell rigidity. The discrepancy between the present study and those showing a decrease in erythrocyte filterability may be due to an effect of chronic smoking, and therefore not evident following acute smoking. However, a difference in filtration systems employed for the measurement of erythrocyte deformability may also explain the different results obtained. Norton and Rand (1981) measured initial flow rate through micropore membranes with pore diameters of 3 μm, whereas a longer filtration time of 6 minutes and membranes with pore diameters of 5 μm were employed for the present studies. The system used by Norton and Rand (1981) would be more sensitive to changes in erythrocyte deformability, but less suitable for measurement of leucocyte deformability (Jones, 1985). The principal reason for the filtration of erythrocyte suspensions was to determine whether the changes in filtration pressures observed with whole blood following smoke exposure were due to an effect of smoke on erythrocyte deformability. Hence erythrocyte suspensions were filtered through 5 μm pores as used for the measurement of whole blood filterability. Also the 5 μm diameter pores are a better representation of the pulmonary capillaries (Weibel, 1963).

CHANGES IN BLOOD VISCOSITY BY ACUTE SMOKING

Changes in plasma viscosity may affect the filtration of blood by altering the rheological properties of erythrocytes (Vejlens, 1938). Although an increase in both blood and plasma viscosity is observed in chronic smokers (Ernst, 1987; Galea, 1985; Rampling, 1991; Rothwell, 1992), acute changes have not been reported (Lowe, 1980). Moreover, the haematocrit was not altered for venous or arterial blood following acute smoking in the present study, and as the blood was diluted for the filtration studies, an increased plasma viscosity would have less influence on filtration.

In summary, a decrease in whole blood filterability was observed for arterial blood sampled following acute cigarette smoking *in vivo*. As blood filterability is believed to reflect the filterability of blood leucocytes, these data suggest acute smoking decreased leucocyte filterability which is consistent with the reduced cell deformability demonstrated for isolated neutrophils in studies of smoke exposure *in vitro* (chapter 4).

6.5.3 DOES SMOKE EXPOSURE CAUSE AN INTRAVASCULAR OXIDANT STRESS?

The data presented in the preceding chapters suggest that the effect of cigarette smoke exposure *in vitro* on neutrophil deformability may be oxidant induced (chapter 5). Several studies have shown changes in blood antioxidant levels following chronic exposure to cigarette smoke (Calder, 1963; Cotgreave, 1987; Galdston, 1984; Joshi, 1988; McCusker, 1990; Pacht, 1986; Toth, 1986). These changes may be in response to the increase in reactive oxygen intermediates released by smokers leucocytes (Ludwig, 1982), but may also reflect a direct increase in the oxidant burden upon the blood induced by acute repeated inhalation of cigarette smoke. Indeed, preliminary data suggest chronic obstructive pulmonary disease, a chronic smoke-related disease, is related to a deficiency in functional plasma antioxidant activity (Taylor, 1986a).
In the circulation leucocytes may be protected from oxidants in cigarette smoke by the antioxidant capacities of plasma, platelets and erythrocytes (section 5.4.1)(Mangione, 1991; Mirabelli, 1989; Pacht, 1986; Toth, 1986). This would explain the unaltered mean whole blood filtration pressures following smoke exposure *in vitro*. However, neutrophils within the pulmonary capillaries, the potential site of exposure to alveolar smoke *in vivo*, are surrounded by very little plasma and few erythrocytes (Bagge, 1976; Skalak, 1989).

Inhaled oxidants need to penetrate through the alveolar air - fluid interface and the alveolar - capillary membrane to reach the capillary blood. However, increased airway epithelial permeability occurs in smokers which may enhance this passage (Huchon, 1984; Jones, 1980; Kennedy, 1984; Nolop, 1987). Inhalation of the potent oxidant ozone is also associated with an increased epithelial permeability (Mustafa, 1990). Moreover, Buckley and colleagues (1975) have shown evidence of an intravascular oxidant stress following ozone exposure in man as measured by a reduction in venous erythrocyte GSH and erythrocyte fragility, and increased lipid peroxidation. Also, morphological changes, consisting of blebs and microvilli-like projections, observed in aortic endothelial cells from rats exposed acutely to the smoke from 8 cigarettes, suggest penetration of cigarette smoke components to the blood stream. although the authors ruled out any endothelial changes by nicotine (Pittilo, 1990).

6.5.4 NO EVIDENCE FOR AN OXIDANT STRESS FOLLOWING *IN VITRO* OR *IN VIVO* SMOKE EXPOSURE

BLOOD GSH LEVELS AS A MEASURE OF OXIDANT STRESS

There was, however, no evidence in the present study of an oxidant stress following acute smoking in either arterial or venous blood samples. GSH is an ubiquitous cellular thiol, and an important antioxidant particularly in erythrocytes (Toth, 1986). That no change in GSH levels in both venous or arterial blood could be detected (Table 6.3), even when 4 cigarettes were smoked consecutively, may be due to the relatively small dose of smoke, and hence oxidant burden, compared with the enormous antioxidant capacity of the erythrocytes (Mangione, 1991; Toth, 1986). However, a reduction in GSH levels was detected following *in vitro* smoke exposure, although higher smoke exposure than normally used for the deformability studies was required.

LIPID PEROXIDATION AS A MEASURE OF OXIDANT STRESS

Similarly, there was no evidence in this study to suggest that polyunsaturated lipids were peroxidised during smoking *in vivo*, as assessed by the levels of the thiobarbituric acid (TBA) reactive product malondialdehyde (MDA) in venous or arterial blood. Increased MDA levels were detected only in the plasma of venous blood samples exposed to cigarette smoke *in vitro* (Figure 6.6), supporting recent *in vitro* work of Frei and colleagues (1991). However, a greater dose (10 puffs) was required before increased levels of MDA could be detected in erythrocytes of venous blood samples. By contrast, Nadiger (1987) and Bridges (1992) and their co-workers did show increased serum MDA levels in smokers compared with non-smokers, but this was probably as a result of chronic exposure.

An increase in MDA concentrations provides a relatively crude index of lipid peroxidation, as discussed in chapter 5. Hence, lipid peroxidation was also determined by measuring the levels of octa-9,11-dienoic acid, the linoleic acid diene conjugate. Measurement of diene conjugates may be preferential to MDA levels as it is a primary product of lipid peroxidation which is retained by all lipid peroxides, whereas MDA is an end product (Halliwell, 1989).

Although an increase in diene conjugated isomers as a ratio of linoleic acid substrate was found for 4 of the 5 samples of blood exposed to smoke *in vitro*, mean values were not significantly altered. Similarly, MacNee and colleagues (1989a) found no increase in octa-9,11-dienoic acid in arterial blood sampled every minute during the course of smoking 1 cigarette.

The method used in this study to measure diene conjugates in plasma, established by Dormandy et al (1987), uses HPLC to separate UV absorbing diene conjugates in human body fluids. Ninety percent of this UV absorbing material consisted of the non-oxygen containing oct-9,11-dienoic acid (Dormandy, 1987). Although peroxidation of biological membranes produces carbon-centered radicals from other fatty acids, not only linoleic acid, and would not be expected to give only a 9[cis], 11[trans] isomer, Dormandy and colleagues (1987) claim that octa-9,11-dienoic acid can be a useful marker of disease activity.

Likewise RBC membrane fragility, as an indirect measure of membrane lipid peroxidation, did not change following smoke exposure *in vivo* or *in vitro*, supporting the data of Norton and Rand (1981). The increased RBC membrane fragility and lipid peroxidation measured in venous blood by Buckley and co-workers (1975) following ozone inhalation was probably due to the longer (2.5 hrs) exposure period in their study, and also because ozone was inhaled with each breath whereas cigarette smoke was "puffed" at intervals. It is thus possible that either the levels of exposure attained *in vivo* in the present study were insufficient to induce lipid peroxidation, or the dilution of exposed with unexposed blood reduced peroxidation products to undetectable levels.

In summary, the data in this chapter confirms that reduced leucocyte deformability occurs following acute cigarette smoking *in vivo*. However, there was no evidence of an oxidant stress in blood during smoking *in vivo*, at least with the degree of smoke exposure used in this study.

CHAPTER 7
SUMMARY AND SUGGESTIONS FOR FURTHER WORK

THE RATIONALE FOR THE WORK IN THIS THESIS

Neutrophils have been implicated in the pathogenesis of several lung diseases, particularly the pulmonary emphysema which develops in association with cigarette smoking (Janoff, 1985). A popular theory of the pathogenesis of this condition is of a proteolytic imbalance in the airspaces causing destruction of alveolar walls and the lesions of centrilobular emphysema. In smokers, neutrophils within the airspaces are believed to be activated by cigarette smoke exposure (Blue, 1978) to release proteolytic enzymes, mainly elastase. Antiproteases in the airspaces bind to and inactivate elastase under normal conditions. However, excess release of elastase by activated neutrophils, and also the inactivation of antiproteases by oxidants in cigarette smoke, can result in an excess of functional elastase which can then enter the interstitium and bind to and destroy elastin, a component of the lung matrix. In addition the oxidative potential of cigarette smoke and phagocyte derived free radicals may directly cause tissue injury.

The concept that a proteolytic insult may occur from the intravascular space has also been considered. MacNee and colleagues (1989d) observed an enhanced sequestration of neutrophils in the lungs during acute cigarette smoking in man. A subsequent study by Bosken and associates (1991) localised this neutrophil sequestration to the pulmonary capillary bed particularly the upper regions of the lungs, the predominant location of centrilobular emphysematous lesions in smokers (Thurlbeck, 1963). Enhanced levels of neutrophil elastase have also been detected in plasma both following chronic (Galdston, 1977) and during acute (Abboud, 1986; MacNee, 1989a) smoking *in vivo*. Furthermore, Bosken and associates (1991) found a transient increase in plasma myeloperoxidase levels as a result of acute smoking. Moreover, a recent preliminary study reported that the neutrophils sequestered within the pulmonary microvasculature were primed to release elastase by cigarette smoke exposure *in vivo* (Hickey, 1993).

Thus, ascertaining the mechanism for the transient sequestration of neutrophils in the lungs during acute smoking could be beneficial in understanding of the pathogenesis of pulmonary emphysema, and hence the development of treatments to modify the disease process in this and other neutrophil-mediated lung diseases.

A MECHANISM FOR THE SMOKE INDUCED PULMONARY SEQUESTRATION OF NEUTROPHILS

In chapter 1 the factors affecting neutrophil pulmonary transit were examined. A change in cell deformability was considered a probable mechanism for the enhanced

neutrophil sequestration and hence investigated with regard to cigarette smoke exposure. Cell deformability was principally assessed by the filtration of a neutrophil suspension through a micropore membrane with dimensions (5 by 11 μm) which are comparable to the average dimensions of the pulmonary capillaries. This *in vitro* measure of neutrophil filtration was shown to correlate strongly with the *in vivo* 'filterability' of neutrophils through the lungs of man, as measured by MacNee et al (1989d)(chapter 3).

A smoke exposure system was devised to allow exposure of cell suspensions *in vitro* to the vapour phase component of cigarette smoke (chapter 2). Pure, quiescent neutrophil populations exposed to cigarette smoke using this system had a markedly reduced cell deformability, associated with a change in cell shape and blebbing of the plasma membrane which indicated cell injury (chapter 4).

DOES CIGARETTE SMOKING ALTER NEUTROPHIL DEFORMABILITY *IN VIVO*?

A transient reduction in the filterability of arterial blood, believed to reflect a reduction in leucocyte deformability, was observed during acute smoking *in vivo* (chapter 6). This change in whole blood filterability was not due to an increase in cell concentration or reduction in erythrocyte deformability. These *in vivo* and *in vitro* studies, in accordance with the studies by MacNee (1989d) and Bosken (1991), propose that impaired cellular deformability was a plausible explanation for the enhanced sequestration of neutrophil in the pulmonary microvasculature during smoking. Further studies to examine the effectiveness of therapeutic interventions which may alter neutrophil kinetics would be beneficial.

A MECHANISM FOR THE REDUCED CELL DEFORMABILITY OF SMOKE EXPOSED NEUTROPHILS

The mechanism by which cigarette smoke alters neutrophil deformability was also investigated. The protection afforded by antioxidants present during *in vitro* cigarette smoke exposure (chapter 5), and the known oxidative potential of cigarette smoke (Pryor, 1983), suggested an oxidant-mediated injury. A similar reduction in neutrophil deformability following incubation with the oxidant hypochlorous acid, and depletion of the ubiquitous intracellular antioxidant glutathione by smoke supported this hypothesis (chapter 5). Furthermore, other components of cigarette smoke, such as nicotine and whole cigarette smoke condensate, which may be less reactive due to its particulate content which adsorb free radicals and the radical

scavenging solvent DMSO in which the condensate was dissolved, did not alter neutrophil deformability (chapter 4). Moreover, the morphological appearance of smoke exposed neutrophils is characteristic of oxidant injury (Mirabelli, 1988a). Such injury may be caused by peroxidative damage to the plasma membrane, resulting in a reduction in the fluidity of the plasma membrane (Rietjens, 1986) which could influence cell deformability. However, lipid peroxidation was not detected at the level of smoke exposure which markedly affected cell deformability (chapter 5). Nor was plasma membrane fluidity significantly different in smoke exposed neutrophils compared with sham exposed cells (chapter 5). However, this study was not conclusive as the lack of significant change in membrane fluidity as a result of smoke exposure may be due to the limited numbers of cells measured in this study. Clearly this is an area which deserves further study. However, in comparison to the viscoelastic interior of the neutrophil, increased rigidity of the plasma membrane is less likely to be a major determinant of cell deformation required for capillary transit.

That no permanent injury was sustained by neutrophils when smoke exposed was supported by the absence of lipid peroxidation. This is consistent with the observed recovery of neutrophil deformability with time, which was enhanced by coincubation of the cells with antioxidants following smoke exposure (chapter 5). The strong antioxidant potential of plasma and erythrocytes could explain the lack a measureable oxidant burden in whole blood following *in vitro* or *in vivo* smoke exposure, unless high doses of smoke were used *in vitro* (chapter 6).

Cell shape change and an increase in cellular stiffness may result from reorganisation of the cell cytoskeleton following smoke exposure (Frank, 1990a; Watts, 1991). A change in filamentous actin (F-actin), a major component of the cell cytoskeleton important for neutrophil shape and motility, was implicated by the improved filterability of smoke exposed neutrophils following addition of the specific F-actin inhibitors cytochalasin B and D (chapter 5). This hypothesis was confirmed by enhanced levels or reorganisation of intracellular F-actin shown by specific fluorescence labelling of the microfilaments with the fluorophore NBD phallacidin, quantitatively and qualitatively assessed by flow cytometry and fluorescence microscopy respectively. In contrast, colchicine, an inhibitor of microtubule formation, did not alter the filtration of smoke exposed neutrophils (chapter 5).

THE EFFECT OF CIGARETTE SMOKE ON NEUTROPHIL FUNCTIONAL BEHAVIOUR

In contrast with most studies of acute and chronic cigarette smoke exposure *in vivo*(Abboud, 1986; Hoidal, 1982; Ludwig, 1982; MacNee, 1989a), diminished functional activity was observed for *in vitro* smoke exposed neutrophils in this thesis (chapter 4). Both the release of oxygen free radicals, and the release and functional activity of the protease elastase from neutrophils was reduced by smoke exposure. This was not due to impaired degranulation as NBT reduction, an endogenous measure of radical release, did not occur in smoke exposed neutrophils, although evident following stimulation of the cells with PMA. However, these *in vitro* studies do not take account of the multiple interactions which can occur *in vivo*, as discussed in chapter 1. Cigarette smoke may interact with other pulmonary cells and their products (Cohen, 1990; Richards, 1992), and also with substances such as glutathione, better known for its antioxidant properties, to result in the production of reactive metabolites with the potential to cause lung tissue injury (Richards, 1992). Moreover, cigarette smoke may induce other resident lung cells, such as the alveolar macrophage and pulmonary epithelial and endothelial cells, to release inflammatory mediators, thereby triggering the sequestered and possibly primed intravascular neutrophils to release a plethora of free radicals and proteases. Indeed alveolar macrophages had increased steady state levels of mRNA for the interleukins IL1α, IL1β and IL6 and platelet derived growth factor (PDGF)(Francus, 1992), and increased synthesis and release of these mediators has also been detected (Francus, 1992; Nagai, 1988; Soliman 1992). Hence, the interaction of cigarette smoke and inflammatory mediators on neutrophil deformability and function would be worthy of further study.

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